सत्यमेव जयते

INTELLECTUAL PROPERTY **INDIA**

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PATENT OFFICE, DELHI
BOUDHIK SAMPADA BHAWAN,
PLOT NO. 32, SECTOR – 14,
NEW DELHI - 110 075.

I, the undersigned being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application, Provisional & Complete Specification*** *and* ***Drawing Sheets*** *filed in connection with Patent Application No.* ***75/Del/2003*** *dated* ***30th January 2003****.*

Witness my hand this 24th day of July 2006.

(P.K. PATNI)
Deputy Controller of Patents & Designs

CERTIFIED COPY OF PRIORITY DOCUMENT

Applicants: Nalam Madhusudhana Rao and Priyamvada Acharya
Serial No.: 10/768,951
Filed: January 29, 2004
Exhibit C

FORM 1

APPLICATION FOR GRANT OF PATENT
(See Sections 5(2), 7, 54 and 135 and rule 33A)

30 JAN 2003

1. We, COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH, Rafi Marg, New Delhi - 110 001, India, an Indian registered body incorporated under the Registration of Societies Act (Act XXI of 1860);
2. hereby declare:
(a) that we are in possession of an invention titled: "Genes Vectors and production of stable lipases"

(b) that the Provisional / ~~Complete~~ specification relating to this invention is filed with this application;
(c) that there is no lawful ground of objection to the grant of patent to us;

3. further declare that the inventor(s) for the said invention is / are:
Nalam Madhusudhana Rao
Ms Priyamvada Acharya are from
Centre for Cellular and Molecular Biology,
Hyderabad India. Both are Indian citizen.

4. We, claim the priority from the application(s) filed in convention countries, particulars of which are as follows: NOT APPLICABLE

5. We state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which we are the applicant:
(a) Patent application no.:
(b) Patent application date:

6. We state that the application is divided out of our application, the particulars of which are given below and pray that this application deemed to have been filed on ..... under section 16 of the Act.
(a) Patent application no.:
(b) Date of filing provisional and / or complete specification ..... and .....

7. That we are the assignee of the true and first inventor(s).

8. That our address for service in India is as follows:

Head, IPM Division, CSIR,
INSDOC Building, 14 Satsang Vihar Marg,
New Delhi - 110 067.

Phone: 696 2560, 696 8819; Fax: 696 8819.

PTO

DUPLICATE

9. Following declaration was given by the inventor(s):

I / We the true and first inventor(s) for this invention declare that the applicants herein is / are my / our assignee:

Dated this 29th day of Jan, ~~19~~/2003

Name (in full with expanded initials)	Signature of the true and first inventor(s)
Nalam Madhusudhana Rao	
Priyamvada Acharya	

10. That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

11. Followings are the attachment with the application:

- ✓ (a) Provisional / ~~Complete~~ specification (3 copies)
- ✓ (b) Drawings (3 copies). 11
- (c) Priority document(s).
- ✓ (d) Statement and Undertaking on FORM-3.
- (e) Power of authority.
- ✓ (f) Fee Rs. 50001- in Cheque no.: 867004 dated: 8.1.03 on State Bank of India, New Delhi Main Branch, Parliament Street, New Delhi – 110 001.

We request that a patent may be granted to us for the said invention.

Dated this 29th day of Jan, ~~19~~/2003

( )
SCIENTIST
Intellectual Property Management Division,
Council of Scientific and Industrial Research.

To,
The Controller of Patents,
The Patent Office, New Delhi.

0075-03

FORM 2

THE PATENTS ACT –1970

PROVISIONAL SPECIFICATION

(See Section 10)

30 JAN 2003

30 JAN 2003

Genes, vectors and

PRODUCTION OF LIPASE

COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH
Rafi Marg, New Delhi –110 001, India, an Indian Registered Body
Incorporated under the Registration of Societies Act (XXI of 1860)

The following specification particularly describes the nature of the invention and the manner in which it is to be performed.

Field of Invention:

The present invention relates to creation, optimization and production of a lipase. More specifically, the invention relates to the generation and production of lipase variants that are thermostable and also stable in organic solvents. The invention also relates to methods of selecting lipase variants for high temperatures and for their purification.

BACKGROUND OF THE INVENTION

Enzymes are the workhorses of a cell that affect virtually every biological process that characterizes a living organism. They catalyze chemical reactions with remarkable specificity and rate enhancements. The awesome catalytic power and versatility of enzymes has long been recognized and enzymes have proved to be very useful outside the living system as well. Enzymes today have widespread application in industry and are seen as environment friendly alternatives to chemical reagents because enzymatic reactions require milder conditions and tend to be cleaner with lesser byproduct and waste generation. Enzymes are being used in numerous new applications in the food, feed, agriculture, paper, leather, and textiles industries, resulting in significant cost reductions and environment-friendly operations.

Enzymes have evolved to function best under the physiological conditions of the parent organism. *In vitro* applications often call upon enzymes to work under non-physiological conditions or to perform functions they have not evolved for. For example, enzymes may have to catalyze reactions involving novel substrates; they may have to work under extreme conditions of salt, temperature, pH etc., or in the presence of potentially inhibiting or denaturing chemicals. Such applications have brought to light the severe disabilities of enzymes to function as industrial catalysts. In order to extract optimum performance from enzymes in

suit specific applications.

The commercial success of these enzymes can be attributed to their ease of use. In addition to functioning at high temperatures, thermostable enzymes generally posses an increased shelf life which markedly improves handling conditions. If enzymes are to play a significant role in large scale processing of chemicals, they must be able to endure the harsh conditions associated with these processes. Thermostable enzymes are easier to handle, last longer, and given the proper immobilization support should be reusable for multiple applications

In obtaining thermostable enzymes the conventional approach is to screen the microbial collections collected from extremephillic environments[1]. The promising candidate enzymes are further investigated for suitability for a specific process. For example, for applications requiring thermostable or salt stable enzymes, enzymes from thermophilic or halophilic organisms were used, respectively. However, such an approach severely restricted the use of enzymes because enzymes for all applications cannot be found in nature. There may not be a natural enzyme for many kinds of transformations. Moreover, enzyme usage is often restricted by undesirable properties of enzymes like product inhibition, low stability etc. Very often an enzyme is required to have a combination of several properties that may be impossible to find in a natural enzyme.

Another approach to obtain thermostable enzymes is based on the current knowledge on the protein structures (crystal structures) of homologous enzymes from mesophiles and thermophiles [2,3]. Such comparisons yielded information on the probable interactions that enhance thermostability. Using such information efforts were made to incorporate these changes in mesophillic enzymes to improve their thermostability. Such approaches have not been very successful since interactions that improve thermostability in a protein are many and each protein acquires, over evolutionary times, those interactions that are best suited for its sequence and the mileu in which it functions. Though structural

determinants of protein stability have been objects of numerous studies on model proteins, no universal stabilization mechanism has yet been found [4]. The most obvious conclusion that can be drawn from the literature is that different proteins have adapted to different thermal environments by a variety of evolutionary devices. The lack of understanding of the structural features leading to protein thermostability has been partly due to a scarcity of data because experimental studies comparing homologous proteins from psychrophilic, mesophilic and thermophilic organisms have been limited to only a few proteins. Moreover, inability to form definite rules for improving protein thermostability is due to the large number and complexity of possible contributing factors [4-7]. Based on comparisons between mesophiles and thermophiles, the main mechanisms responsible for increased thermostability have been identified as increase in the number of hydrogen bonds and salt bridges, increased core hydrophobicity, better packing efficiency, α-helix and loop stabilization and resistance to covalent destruction. Often it becomes difficult to delineate protein interactions that contribute to thermostability from other selection pressures such as salt, pH etc.

Other strategies adapted to increase the thermostability was based on the observation that immobilized enzymes acquire thermostability to some extent [8]. Hence, several solid supports were tried to immobilize proteins. And also recent observations made with enzymes in organic solvents indicated that in organic solvents enzymes acquire thermostability [9]. The advent of recombinant DNA techniques has greatly facilitated protein engineering by allowing facile mutagenesis and production of proteins.

The term protein thermostability refers to the preservation of the unique chemical and spatial structure of a polypeptide chain under extremes of temperature conditions [4]. In general, the higher the temperature to which the enzyme is exposed, the shorter the half-[illegible] the enzyme (i.e., the shorter the enzyme retains its activity). Similarly, the greater levels of organic solvent to

which said enzymes are exposed, the shorter the half-life of the enzyme. The phrase "catalytic activity" or simply "activity," means an increase in the k.sub.cat or a decrease in the K.sub.M for a given substrate, reflected in an increase in the k.sub.catt /K.sub.M ratio.The structural basis of protein thermostability has been an actively pursued area of research for at least two decades [10] . However, enzymes lifted out of the context of living organisms do not always function as well as they do in their natural milieu. For example, they have optimum activity at the physiological temperature of the organism and tend to denature at higher temperatures leading to drop in activity. Thermostable enzymes are important as they can be used at high temperatures and harsher conditions required in industrial contexts. Also they generally have higher storage stabilities and bring down costs by obliviating the need for low temperature storage and decreasing the loss due to denaturation on storage and handling. Moreover reactions carried out at higher temperatures generally proceed at higher rates further bringing down operation times.

In view of the environmental safety reasons, there is a constant pressure to reduce the use of environmentally polluting processes in industry. Enzymes are increasingly used to replace chemical processes in leather, food, and pharmaceutical industries. Comparison of protein structures from extremeophiles demonstrated that protein structural plasticity is enormous and is resident in the primary structure. This lent considerable support to strategies that alter the primary structure of the proteins at the genetic level and screen for the variants with special properties such as thermostability. The tremendous success in handling the genes and developing protocols to alter it at will, has allowed to evolve proteins with special functions. The strategy relies in generating variation in gene sequences by molecular biology methods and screening the variants by expressing them and screening the mutant population [11-13]. The screening protocols are based on the property of interest, e.g., activity at high temperature or activity in the presence of organic solvents. The present

invention encompasses methods for generating variation in gene sequences, protocols for screening the enzymes with higher thermostability and also protocols for sequencing and expression.

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of ester bonds in triacylglycerols and often also exhibit phospholipase, cutinase and amidase activities [14]. They are used for the production of detergents, pharmaceuticals, perfumes, flavour enhancers and texturising agents in consmetic products. Lipases are crucial for the production of a wide variety of foods, especially for products from milk, fat and oil. Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases [15] . Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides and other chemicals. An increasing number of lipases with suitable properties are becoming available and efforts are underway to commercialize biotransformation and syntheses based on lipases [16]. Enzyme sales for use in washing powders still remain the single biggest market for industrial enzymes. The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year Because of their ability to hydrolyzes fats, lipases

find a major use as additives in industrial laundry and household detergents. Detergent lipases are especially selected to meet the following requirements: (1) a low substrate specificity, i.e., an ability to hydrolyze fats of various compositions; (2) ability to withstand relatively harsh washing conditions (pH 10–11, -60 _C); (3) ability to withstand damaging surfactants and enzymes [e.g., linear alkyl benzene sulfonates (LAS) and proteases], which are important ingredients of many detergent formulations. Lipases with the desired properties are obtained through a combination of continuous screening [17-19] and protein engineering [20]. In 1994, Novo Nordisk introduced the first commercial recombinant lipase 'Lipolase,' which originated from the fungus Thermomyces lanuginosus and was expressed in Aspergillus oryzae. In 1995, two bacterial lipases were introduced— 'Lumafast' from Pseudomonas mendocina and 'Lipomax' from P. alcaligenes— by Genencor International [15]. According to a report an alkaline lipase, produced by P. alcaligenes M-1, which was well suited to removing fatty stains under conditions of a modern machine wash. The patent literature contains examples of many microbial lipases that are said to be suitable for use in detergents [22].

Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomyces. Bacillus subtilis is a Gram-positive, aerobic, spore-forming bacterium that has generated substantial commercial interest because of its highly efficient protein secretion system Though extracellular lipolytic activity of B.subtlis was observed as early as in 1979 [23], molecular research started in 1992 when a lipase gene, lipA, was cloned and sequenced [24]. Subsequently the lipase was overexpressed, purified and characterized [25]. Later, a second gene, lipB, was found that is 68% identical with lipA at the nucleic acid level [26]. This gene has been cloned and the protein overexpressed, purified and characterized.

The *Bacillus subtilis* lipase with a molecular weight of 19,348 Da is one of the smallest lipases known. It is one of the few lipases that do not show the interfacial activation in the presence of oil-water interfaces. LipA is very tolerant

glycerol esters with both short and long chain fatty acids, showing optimum activity with C8 fatty acid chains.

Bacterial lipases are classified into eight families according to their sequence similarities, conserved sequence motifs and biological properties [27]. The true lipases are classified in family I which contains six subfamilies. Bacillus lipases have been placed in subfamilies 4 and 5. In these two subfamilies alanine replaces the first glycine residue in the conserved G-X-S-X-G pentapeptide around the active site serine residue. Subfamily 4 consists of only three members, LipA and LipB from B.subtilis and a lipase from *Bacillus pumilis*, which share 74-77% sequence identity. These are the smallest lipases known and show very little sequence similarity (~ 15%) with the other, much larger, Bacillus lipases that constitute subfamily 5.

The crystal structure of the B.subtilis lipase LipA reveals a globular protein with dimensions of 35 X 36 X 42 [28]. The structure shows a compact domain that consists of six β- strands in a parallel β- sheet, surrounded by α- helices. There are two α- helices on one side of the β- sheet and three on the other side. The fold of the B.subtilis lipase resembles that of the core of the α/β hydrolase fold enzymes. The B.subtilis lipase lacks the first two strands of the canonical α/β hydrolase fold and the helix αD is replaced by a small 310 helix. The helix αE is exceptionally small, with only one helical turn, and several α-helices start or terminate with 310 helical turns. Due to these structural features, its small size and absence of a lid domain, the B.subtilis lipase is considered a minimal α/β hydrolase fold enzyme.

Description of the invention:

In accordance with the present invention a methods of directed evolution were applied to the lipase gene (Gene Seq ID # 1) to isolate protein variants of the original sequence which possess increased thermostable properties. The methodology relies initially on the ability to create random variations in the original gene sequence and express the corresponding proteins in the bacteria, E.coli. The produced variants of the original sequence would have altered sequence, hence altered properties. The variants, at a proteins level , would be tested for their thermostability and those sequences which demonstrate improved thermostability would be subjected to the next round of random mutagenesis and screening. Thus by sequential accumulation of the mutants and subsequent pooling of the mutations the thermostability of lipase was improved by 200-fold at high temperature. High temperature range includes temperature ranges from 50-90 C.

The method according to the present invention includes four steps in generating the variant lipases and their characterization to obtain thermostable lipases. In the first of the methods variation in the primary sequence of the lipase gene were generated by error prone PCR methods. The adapted protocols are similar to several published protocols. In addition many of the different protocols such as random priming, ITCHY etc could also be applied for generation of variance in the gene sequence [29,30]. In the second step of the invention, the mutant sequences are cloned into an expression vector and the protein expressed in the culture lysates. The expressed proteins are screened for their ability to withstand higher temperature was tested in a large population using medium-throughput methods. In the third step of the invention the promising variants were pooled by a family shuffling procedures according to the method of Stemmer [12] and furth · tested for thermostability. .. addition to the shuffling p. cedures selective changes in the primary sequence were also incorporated by standard

molecular biology procedures. In the fourth step of the invention the positive sequences were over-expressed in large cultures and the proteins are purified by the published procedures according to Colson et al [24]. The purified proteins were tested for their thermostability.

The screening procedures of the lipase variants for increased thermostability involve ability to hydrolyse chromogenic substrate esters based on p-nitrophenyl group. Natural substrates of lipase are triglycerides, which are not convenient to design a simple medium trough put assays wherein the source of enzyme is over-expressed lipase in a cell lysate [31]. P-nitrophenyl esters of fatty acids are convenient and, the activity of lipase represents the activity of lipase on triglycerides. Long chain esters of p-nitrophenyl, especially p-nitrophenyl oleate, are well suited for this purpose. Detergent solubilized PNPO demonstrates negligible back ground hydrolysis and well suited for lipase present in cell lysate. The strong yellow color of very high extinction coefficient of hydrolysis product p-nitrophenyl can be estimated conveniently in a 96-well plates. Along with p-nitrophenyl esters, many other fluorogenic or chromogenic esters of fatty acids could be used for this purpose.

As employed herein the term thermostability refers to the property of the enzymes, which retain their activity subsequent to exposure to higher temperatures. Enzymes lose their tertiary conformation on exposure to higher temperature due to the increased movement of the structural elements, which perturbs the functional structure of the protein. Typically proteins lose their activity at higher temperatures with time. The rate of this loss in activity, reflects in half life i.e., time required to lose half of the initial activity, is a convenient parameter to compare the thermostability of the protein [4]. Activity, as defined here, corresponds to the catalytic acitivity represented by the term kcat/Km, where kcat is the rate of the product formation and Km is the apparent affinity constant of the substrate to the enzyme. Retaining the functional structure at

elevated temperatures resides in the ability to form interactions within the protein that withstand high temperatures. The range of the temperature that is relevant for the present invention ranges from 35 to 90 C.

The naturally occurring lipase from Bacillus lipase has the amino acid sequence of 1-181 as given in the sequence ID # . The corresponding nucleic acid sequence expressing the protein (ID #)was presented in ID # . It was discovered that the amino acid substitutions at positions 68, 71, 114, 120, 132, 144, 147 and 166 were found to be important for the thermostability of the lipase. In accordance with the present investigation, it was further discovered that the substitutions at positions 114, 132 and 166 are suited for increasing the stability of the proteins. Any of the innumerable combinations of substitutions possible at each of these positions with the other 19 amino acids would be favourable for the thermostability.

The specific substitutions of relevance for thermostability in lipase are given below.

From	To	Position
N	V	166
A	D	132
A	V	68
L	P	114
R	S	147
V	A	144
N	D	120

Examples:

Example 1:

Purification of lipase from Bacillus subtilis

Purification of the lipase was performed from E.coli cells expressing the lipase in an appropriate vector. The purification essentially involves passing the cell lysate in phenyl-sepharose column followed by a Mono-S column. Lipase is an aggregated prone protein, care especially keeping protein concentration below 5mg/ml, was taken to avoid aggregation of the protein. The purification of the lipase was carried out essentially as described earlier[32] with minor modifications. Lipase from culture filterates of Bacillus strain or from the E.coli lysates was processed similarly. For purification of the wild type and mutant proteins from E.coli the lipA gene or the mutant genes are cloned into pET 21b.). For this, the gene corresponding to the full length, mature protein was amplified with primers PrNde I (forward primer) (5'-CCATGATTACGCATATGGCTGAACACAA-3') and JOF. The forward primer had an engineered Nde I site. The forward primer also introduced a start codon at the start of the lipase gene in the form of the ATG sequence that is part of the Nde I recognition sequence. This would introduce a methionine in the N-terminus of the mature protein, expressed in *E.coli*, just before the N-terminal alanine that occurs in the protein purified from the culture supernatant of *B.subtilis*. The wild type protein as well as the mutants were amplified, digested with Nde I and BamH I and ligated with pET-21b digested with Nde I and BamH I. The ligation mix was transformed into *E.coli* DH5α and the positives were selected by plasmid minipreps and restriction digestions (Fig.1).

Protein was purified from *E.coli* BL21 (DE3) cells. Cells containing the appropriate plasmid were grown till mid-log phase before inducing with 0.5 mM IPTG. Cells were harvested 2.5 hours a[illegible] duction by centrifuging at 15,000 rpm at 4 °C for 20 min. The pellet was washed with STE and resuspended in 1X

TE containing 0.3 mg/ml lysozyme. The suspension was incubated on ice for 30 min before lysing the cells by sonication. Sonication was carried out by keeping the cells on ice. Short pulses of half-minute duration were applied and 1 min cooling time was allowed between pulses. The sonicated cells were centrifuged at 20,000 rpm at 4 °C. The supernatant was loaded on a phenyl sepharose column. The remaining steps were done as described in chapter 2. The purified proteins were stored in -70 °C till further use.

B.subtilis BCL1051 was grown aerobically for 16-18 hrs at 37 °C in 2l Erlenmeyer flasks, each containing 500 ml of medium of the following composition: 2.4 % yeast extract, 1.2 % tryptone, 0.4 % gum Arabic, 0.4 % glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4, 50 mg/ml kanamycin sulfate. The culture medium was inoculated at 1 % from 10 ml precultures. After harvesting the cells by centrifugation at 6000 rpm for 30 min, the culture supernatant was pumped at a flow rate of 30 ml/hr onto a Phenyl Sepharose Fast Flow High sub column (Pharmacia) (20 ml column volume per 1l culture) equilibrated with 100 mM potassium phosphate, pH 8.0. The column was washed at a flow rate of 50 ml/hr first with 10 mM potassium phosphate, pH 8.0 and then with 30% ethylene glycol in 10 mM potassium phosphate, pH 8.0. Elution was performed at a flow rate of 50 ml/hr with 80 % ethylene glycol in 10 mM potassium phosphate, pH 8.0. 2 ml fractions were collected and the fractions containing protein (detected by absorbance at 280 nm) were checked for enzyme activity. The active fractions were pooled and dialyzed against 2 mM glycine-NaOH, pH 10.0. The dialyzed protein was diluted 1:1 with 50 mM Bicine-NaOH, pH 8.5 (buffer A) and loaded onto a MonoS HR5/5 (Pharmacia) column, pre-equilibrated with buffer A, using a Superloop (Pharmacia) on a FPLC (Pharmacia) system. The protein-bound-column was washed thoroughly with the buffer A to remove unbound proteins. The protein was eluted using a linear gradient with buffer A to buffer B (50 mM Bicir NaOH, pH 8.5, 1 M NaCl). Thc nzyme eluted around 300 mM aCl as a single peak. The active fractions eluted from the MonoS column were dialyzed

overnight against 2 mM glycine, pH 10.0 and concentrated using an Amicon concentrator fitted with a YM10 membrane (10 kD cutoff). Purity of the protein was checked on a 12% SDS-PAGE gel containing 5 M urea (Lessuisse *et al*, 1993). The protein was > 95 % pure on a Coomassie stained gel (Fig.2).

Example 2:

Assay of lipase:

Lipase belongs to a class of enzymes known as interfacially active enzymes. These enzymes have very little activity on the substrate monomers but their activity increases dramatically on insoluble substrate such as emulsified triglycerides, monolayers etc. This property makes lipases dissimilar to other enzymes which act on soluble substrate monomers. Triglycerides, natural substrates of lipase are not very convenient to set up simple chromogenic assays (Fig.3). Activity of pure lipases, sometimes, can be monitored by detecting the pH changes using pH-sensitive dyes. However, such assays yield complications when the enzyme source is a lysate and when there are other processes that may alter the pH. P-nitrophenyl esters are most convenient to monitor the activity. Short chain ester, p-nitrophenyl acetate and long chain ester, p-nitro phenyl oleate (PNPO), were synthesized by routine synthetic methods (given below). PNPO is a insoluble ester, was used in our assays using triton X-100 as a solubilizing agent. Triton X-100: PNPO co-micelles showed low back ground hydrolysis and were also stable at elevated temperatures. 96-well plate assays for screening the variants of lipase, though very useful to screen large number of samples, quantitates the activity approximately. All positives obtained in 96-well screens were confirmed in a tube assays, where the number of samples are less and more accurate specific activity calculations could be made.

Synthesis of chromogenic substrates for lipase assays

The following chromogenic substrates were synthesized for lipase assays:

1) p-nitrophenyl oleate
2) p-nitrophenyl stearate

3) p-nitrophenyl caprylate

The fatty acid, N, N'- methyltetrayl biscyclohexamine (dicyclohexylcarbodiimide, DCC), N, N'- dimethylamino pyridine (DMAP), and p-nitrophenol were taken in mole ratios of 1:1:1:2. The fatty acid was taken in a round bottom flask containing 20 ml of dry DCM and a few ml of chloroform. The mixture was stirred for two minutes followed by addition of DCC. A white precipitate was formed. This was followed by the addition of DMAP. Subsequent addition of p-nitrophenol led to the formation of a yellow precipitate. The reaction vessel was flushed with nitrogen and stirred for 5 hours. The progress of the reaction was monitored by thin layer chromatography. After completion of the reaction, the DCM was evaporated to dryness and the ester was purified by column chromatography (silica gel column, elution with petroleum ether-acetone). The purity and identity of the product was confirmed by ^{1}H-NMR spectroscopy.

Example 3

Lipase assay in 96-well microtitre plates): The colonies obtained from the cloning of the PCR product generated by error-prone PCR were patched on another similar plate and simultaneously inoculated in separate wells of a microtitre plate containing 200 µl 2XYT containing 25 µg/ml chloramphenicol and 0.2 % glucose. The cells were grown for 24 hours in the microtitre plate with continuous shaking at 200 rpm. After 24 hrs, 5 µl culture from each well was taken and added to the corresponding well another microtitre plate containing 200 µl 2XYT supplemented with 25 µg/ml chloramphenicol. After 3 hours of growth the cultures were induced with 1 mM IPTG. After 3 more hours 25 µl of culture was taken from every well into the corresponding wells of two fresh microtitre plates containing 25 µl phosphate buffer pH 7.0. One of the plates was exposed to high temperature for 20 min, cooled on ice for 15 min and then allowed to come to room temperature. The other plate was kept at room

temperature. 25 µl of the Triton X-100 substrate solution prepared as described above was added to each well. The plates were incubated at 37 °C and absorbance at 405 nm was recorded in an ELISA reader at definite time intervals. The clones showing less than 20 % of the activity of the wild-type protein (or the parent from which it is generated) were removed from further consideration. The residual activity for each clone after exposure to high temperature was calculated. The clones showing highest residual activity were chosen for the next level of screening.

Lipase assays in tubes:: The colonies that showed highest residual activity in the microtire plate level screen were grown for 12 hours in 5 ml 2XYT medium 25 µg/ml chloramphenicol and 0.2 % glucose. 10 ml of 2XYT containing 25 µg/ml chloramphenicol and 0.2 % glucose was inoculated with 100 µl of the overnight grown culture. After 2.5 hours growth, the cultures were induced with 1.5 mM IPTG and were harvested after another 2.5 hours. The cell pellet was washed with STE and resuspended in 1 ml 0.05 M potassium phosphate buffer pH 7.2. The cell suspension was sonicated with a Branson sonicator with four pulses of 30 sec and 1 min cooling time in between the pulses. The tubes were kept on ice during sonication and cooling of the samples. The sonicated samples were centrifuged at 15,000 rpm for 45 min and the supernatant was used for the assays. The supernatant was divided into four 250 µl aliquots. Three of the aliquots were exposed to higher temperatures and the fourth was kept on ice. The tubes were exposed to high temperatures for 20 min, chilled on ice, centrifuged at 4 °C at 15,000 rpm and then allowed to come to room temperature before assaying for enzymatic activity. The lipase activity in the cell lysates was determined at room temperature in sodium phosphate buffer pH 7.2 by using p-nitrophenyl oleate as substrate. The enzymatic activity was measured by following the change of absorbance at 405 nm with time. Lysates of cells that do not contain the lipase gene but other processed in the same way as mentioned above, were used to determine the background hydrolysis of p-

nitrophenyl oleate in *E.coli* cell lysate. The background hydrolysis values were subtracted from the enzymatic activity value. The total protein in the cell lysates was determined by Lowry's method and was used to normalize the activity.

Example 4

Half-lives of thermal inactivation

Exposing the enzymes to higher temperatures and then assaying the activity at room temperature normally assess thermostability of enzymes. At higher temperature the protein denatures and irreversible unfolds. Thermostable enzymes possess additional stabilizing interactions which would make them less susceptible for heat denaturations. The activity remaining is residual activity, which decrease both with increase in temperature or with increase in time at a given temperature. Heat treatment of the purified proteins was carried out in a programmable thermal cycler (GeneAmp PCR system 9700) in 0.2 ml thin-walled PCR tubes to allow precise temperature control of the samples. The proteins were taken at a concentration of 0.05 mg/ ml in 0.05 M sodium phosphate buffer, pH 7.0. 25 µl of protein samples were taken in each tube. The proteins were heated for the required time, cooled at 4 °C for 20 min, centrifuged and equilibrated at room temperature before assaying for enzymatic activity. 20 µl of the heat-treated protein sample was added to 1 ml 0.05 M sodium phosphate, pH 7.2 containing 2 mM p-nitrophenyl acetate. Enzymatic activity was measured at 25 °C by monitoring the rate of increase in absorbance at 405 nm. Typically, inactivation was followed until > 80 % of the activity was lost. Plots of log(residual activity) versus time were linear. Inactivation rate constants (k_{inact}) were obtained from the slope and half-lives were calculated as $t_{1/2} = \log 2 / k_{inact}$. The half lives of various mutants obtained were presented in figure (Fig.4) where the residual activities were measured using PNPA as substrate. The enzyme mutants were exposed to 55 C. In fig.5 data obtained with residual activities with three mutants using olive oil as a substrate was presented. The activities were measured using pH stat equipment. This data demonstrates that the

enhancement seen with mutants was independent of the substrate and nature of the assay.

Preparation of substrate stocks:

Appropriate amounts of the insoluble p-nitrophenyl ester and Triton X-100 were weighed out in a glass vial and mixed with a magnetic stirrer till the ester completely dissolved in Triton X-100. Buffer was added slowly while stirring to prepare a 2X stock solution containing 0.4 mM p-nitrophenyl ester and 40 mM Triton X-100. Substrate solutions prepared in this way were optically clear. 100X substrate stocks of the water-soluble p-nitrophenyl acetate were made in acetone and 2 mM p-nitrophenyl acetate was used for each reaction. The reactions were carried out in absence of Triton X-100 and all the measurements to determine kinetic parameters were done with this reaction system.

Example 5

Assay with olive oil

Assay with the olive oil is performed pH stat equipment. All lipases subsequent to thir activity reduce th pH off the reaction medium by releasing a proton. The decrease in pH could be neutralized by addition of known amounts of alkali. The rate of additon of alkali would represent the activity of the lipase. We have prepared the lipase substrate by mixing gum Arabic (0.5%), olive oil ( ) and CaCl2( ). The mixture was sonicated in a bath till we obtain a uniform emulsion. We have used 10 ml of the substrate for each assay.At the beginning of the asay the pH of the substrate was brought to 8.4 by addition of alkali. The reaction was started with the addition of 10 microlitres of 1mg/ml enzyme solution. The rate of reaction was calculated from the slopes of amount of alkali vs. time curves.).1 N NaOH was sued as alkali.

Example 6

ethods of generation of variations in the Lipase genes:

The sequence of LipA, whose product is lipase gene of interest in this invention, from *Bacillus subtilis* was published. In Bacillus LipA gene product is secreted into the culture medium owing to the presence of a signal sequence at the N-terminal of the sequence, which aids in its transport out of the cell. Molecular biology of Bacillus species has been well studied and it is a Gram-positive strain. For routine molecular biology techniques such as transformation, cloning, expression etc. Bacillus sp. is less suited compared to E.coli [33,34]. The main difficulty is in transforming the Bacillus sp with the plasmids. The efficiency is lower by several orders of magnitude compared to E.coli. Further, the observed efficiencies are only detectable with electroporation, which is a harsher method. In E. coli the transformation efficiency is higher and reproducible and the choice of plasmids is wide. To perform various gene manipulations, E.coli was used.

The clone pLipA containing the complete lipA gene in pBR322 plasmid was a kind gift from Dr Frens Pierce (Fig.6). The lipase gene along with the region coding for the signal sequence was amplified with primers For1 (forward primer) (5'-GGAGGATCATATGAAATTTGTAAAAA-3') and Rev1 (reverse primer) (5'-CCCGGGATCCATTGTCCGTTACC-3'). The primers contained engineered Nde1 and Bam H1 sites respectively. The ATG of the Nde1 site in For1 coincided with the natural start codon of the lipase and the BamH1 site was beyond the natural stop codon. The amplified product was digested with Nde 1 and Bam H1 and cloned into the Nde 1-BamH1 sites of the plasmid pET-21b yielding the plasmid pET-lipwt (Fig.7) . The lipase gene coding for the mature protein was amplified from pET-lipwt by using primers PREcoR I (forward primer) (5'-CGTCAGCGAATTCCGCTGAACACAT-3') and PRBamH I (reverse primer) (5'-GCGGGAAGGATCCGAATTCGAGCT-3'). The primers had an engineered EcoR I and BamH I site respectively. The amplified product was cleaved by EcoR I and BamH I and cloned into the EcoR1-BamH1 sites of the plasmid pJO290. This construct (pJ0290lip) was used for screening thermostable mutants (Fig.8). The *E.coli* strain JM109 was used for all the screening steps and all media contained

0.2 % glucose unless otherwise mentioned. This system was chosen because it allows low-level, controlled and inducible expression of the gene product in *E.coli*, which is necessary to prevent the reported toxicity of the protein to *E.coli* and to prevent complications from *in vivo* insolubility of this highly hydrophobic and aggregation-prone protein.

Methods of random mutagenesis:

The critical step in the invention is in the ability to create variations in the gene. The variation generated should be "sufficient' to yield functional variants. Enzymes have evolved over millions of years of evolution and in the process the enzymes may have tested and avoided deleterious mutations and also tested and incorporated beneficial mutations. It is also believed that most of the gene mutations would be silent i.e., they do not bring about a change in amino acid sequence. In random mutagenesis protocols, it is essential to obtain variations in the gene sequence that result in non-silent mutations and excess of variations, wherein the gene product would be non-functional or may not form. Error-prone PCR based mutagenesis protocols need to be optimized to obtain sufficient variation in the activity of the lipase. The success of the directed evolution protocols strongly depends on the control of this variable. The protocols used in the present example were modifications of the published procedures.

The lipase gene was mutagenised by error-prone PCR (Cadwell and Joyce, 1992). Primers JOF (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and JOR (5'-TGACACAGGAAACAGCTATGAC-3') flank the gene beyond the Eco R1 and Bam H1 sites present on the plasmid. Error-prone PCR was carried out in a 100 µl reaction volume containing 20 femtomoles of the plasmid pJO290-lip, 50 pmoles each of primers JOF and JOR, 100 mM Tris.Cl (pH 8.3 at 25 °C), 500 mM KCl, 0.1 % gelatin (w/v), 7 mM $MgCl_2$, 0.25 mM $MnCl_2$, 1 mM each of dTTP and dCTP, 0.2 mM each of dATP and dCTP and units Taq DNA polymerase. After an initial denaturation of 3 min at 94 °C, the following steps were repeated for 30

cycles in a thermal cycler: 1 min at 94 °C, 1 min at 45 °C and 1 min at 72 °C. The amplified product was precipitated with ethanol, eluted from a 1 % agarose gel and digested with EcoR I and BamH I. The digested product was again eluted from a 1 % agarose gel and ligated with pJO290 digested with EcoR I and BamH I. The ligation mix was transformed into *E.coli* JM109 and selection was done on LB-agar supplemented with 25 µg/ml chloramphenicol and 0.2 % glucose.

Site-directed mutagenesis

Site directed mutagenesis was carried out on the lipase gene cloned in pET-21b by a modified PCR technique (Chen and Arnold, 1991). For each substitution an oligonucleotide containing the desired mutation was used as the primer (mismatch primer) to initiate chain extension between the 5′ and 3′ PCR primers. In the first PCR, the mismatch primer and the 3′ primer were used to generate a DNA fragment containing the new base substitution. The fragment was separated from the template and primers by agarose gel electrophoresis, purified and used as the new 3′ primer in a second PCR with the 5′ primer to generate full length product, which was cloned into pET-21b for expression of the mutant protein.

Example 7

Recombination of the clones obtained in generation 2

The mutant 3B1 was created from the clone 2-8G10 and wt by using the unique restriction site Hae II at position 910 of the lipase gene. The genes coding for the two proteins were amplified by PCR using the T7 promoter and terminator primers. The PCR products were purified by gel extraction and digested with Hae II and Nde I. The upper and lower bands correspond to the C-terminal and N-terminal regions of the protein, respectively. The upper band from clone 2-8G10 and the lower one from the wild-type protein were eluted. The higher molecular weight fragment was digested with BamH I and purified. A three point ligation containing the Nde I- Hae II fragment (from the wt), the Hae II-

BamH I fragment (from 2-8G10) and pET-21b cut with Nde I and Hae II was set up, the ligation mix transformed into DH5α and the positives selected (Fig.9). The sequence of the gene was confirmed by DNA sequencing.

The mutant 4B1 (triple mutant) was created by site-directed mutagenesis on the 3B1 template using the mutagenic primer PROLF: 5'- GGC AAG GCG CCT CCG GGA ACA GAT- 3' to incorporate a codon change CTT → CCT that led to L114P change in the amino acid sequence. The sequences of all the genes were confirmed by automated DNA sequencing.

Example 8

Enzyme kinetics

All kinetic measurements were made using a thermostatted spectrophotometer using the water-soluble substrate p-nitrophenyl acetate. Initial rates of hydrolysis of p-nitrophenyl acetate at various concentrations were determined at 25 °C in sodium phosphate buffer pH 7.2. The values for K_M and k_{cat} were derived from the corresponding Lineweaver-Burke plots. The kinetic parameters obtained with wild type and the mutants was presented in fig. 10.

Example 9

Activity of lipase and its mutants in the presence of organic solvents

The activity of the lipasea nd its mutants was checked in the presence of various solvents. The organic solvents tested were acetonitrile, isopropanol, dimethyl sulfoxide and dimethyl formamide. The activity assay was performed using PNPA as a substrate. The substrate (2 mM) was dissolved in various percents (v/v) of the organic solvent in buffer (50 mM pH 8.0) and the reaction was started with the addition of lipase at a concentration of 0.246 mg/ml. The activity was monitored as n increase in absorption at 410 nm and the specific activity was calculated using the initial slopes of the curve. In fig 11 the data obtained with acetonitrile is presented.

The preceding examples demonstrate the usefulness of the present invention in generating, identifying and isolating lipases which have improved stability and/or ester hydrolysis activity at higher temperature in organic media relative to the natural enzyme.

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the disclosures herein are exemplary only and that various other alternations, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein.

Description of figures

Fig.1 : Subcloning of lipA without the signal sequence into pET 21b

Fig.2. SDS-PAGE profiles of the purified proteins. All the lipases were purified by the procedures given in examples. Lane 1: Low Molecular weight marker, 2: wild-type lipase, 3: 1-1E5, 4: 2-8G10, 5: 2-3H5, 6: 3B1, 7: 4B1

Fig.3 Hydrolysis of a triglyceride catalyzed by a lipase

Fig.4: Residual activity of various mutants and the wild type at a various times on exposure to a temperature of 55 C. The substrate used is PNPA.

Fig.5 Residual activity of various mutants and the wild type at various times on exposure to temperature of 50 C. The substrate used is olive oil.

Fig.6 The lipA gene pBR 322

Fig.7 Subcloning lipA along with the suignal sequence into pET 21b

Fig.8 Subcloning of lipA without signal sequence into pJO290

Fig.9 Recombination of the clones obtained in generation II

Fig.10 Kinetic parameters and half life of stability at 55 C of wild type and mutant lipase.

Fig.11 Activity of lipase and its mutants in the presence of acetonitrile at various concentrations in water.

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Fig.1

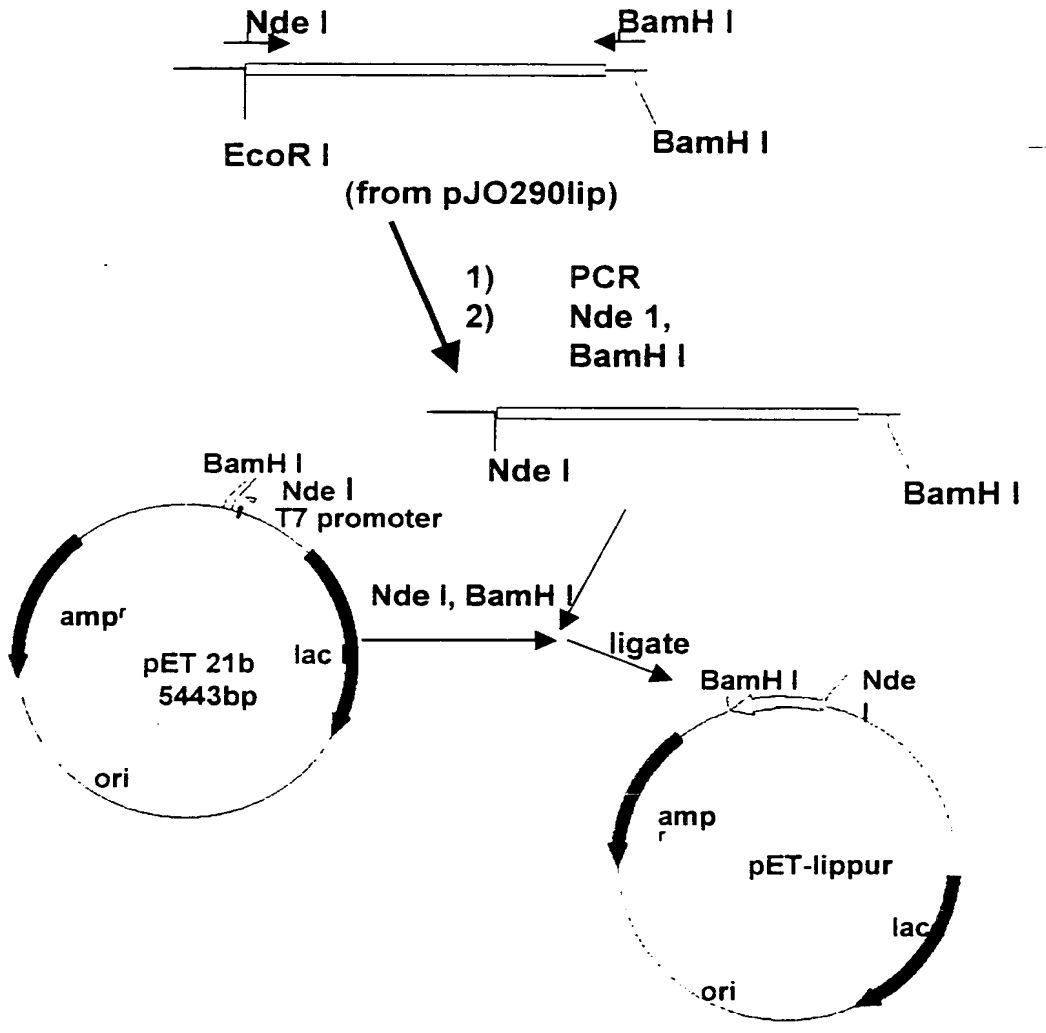

Nde I
BamH I
EcoR I
BamH I
(from pJO290lip)
1) PCR
2) Nde 1, BamH I
Nde I
BamH I
BamH I
Nde I
T7 promoter
amp r
pET 21b
5443bp
lac I
ori
Nde I, BamH I
ligate
BamH I
Nde I
amp r
pET-lippur
lac
ori

Fig.2

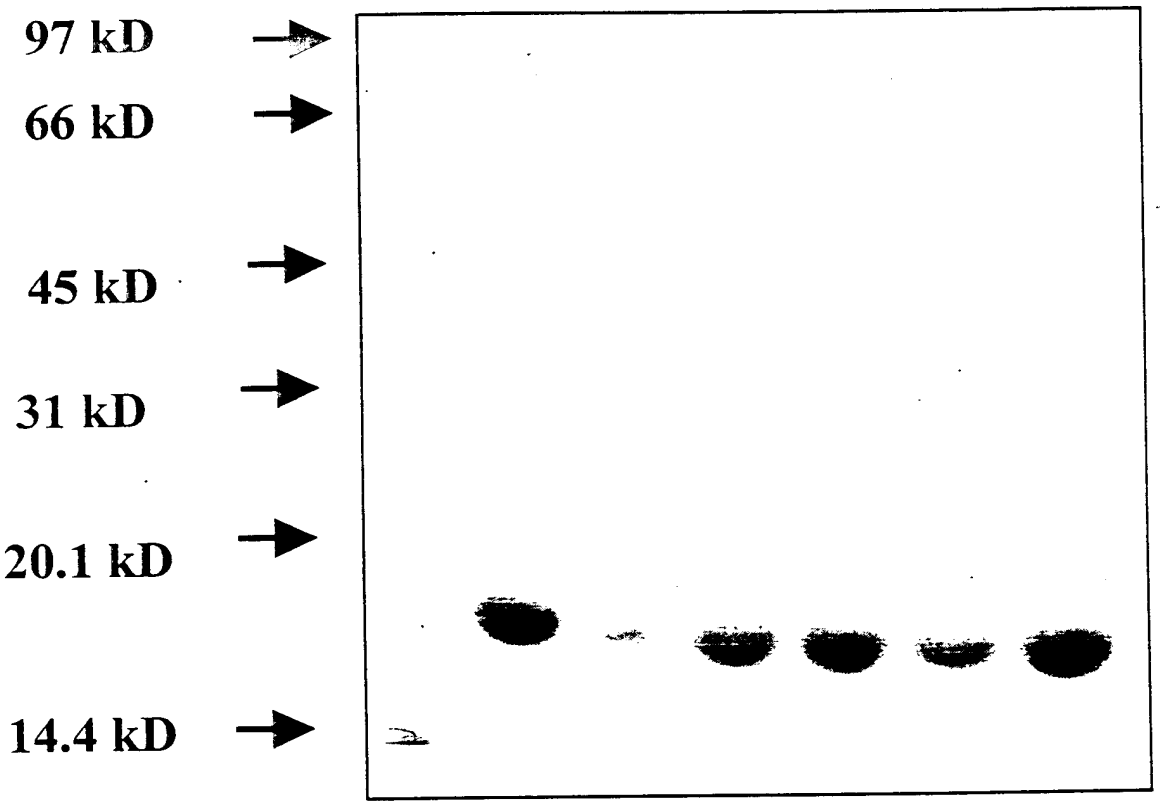


Fig.3

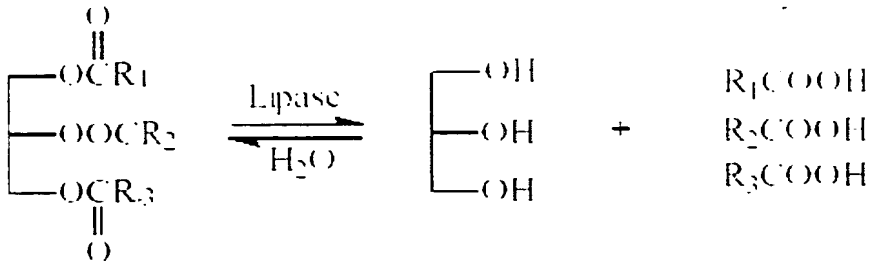


Fig.4

The residual activity of the proteins wt (○), 1-1E5 (▲), 2-8G10 (◆), 2-3H5 (□), 3B1 (●), 4B1(■) plotted against the time of exposure at 55 ° C, pH 7. The experiment was carried out as described in Materials and methods.

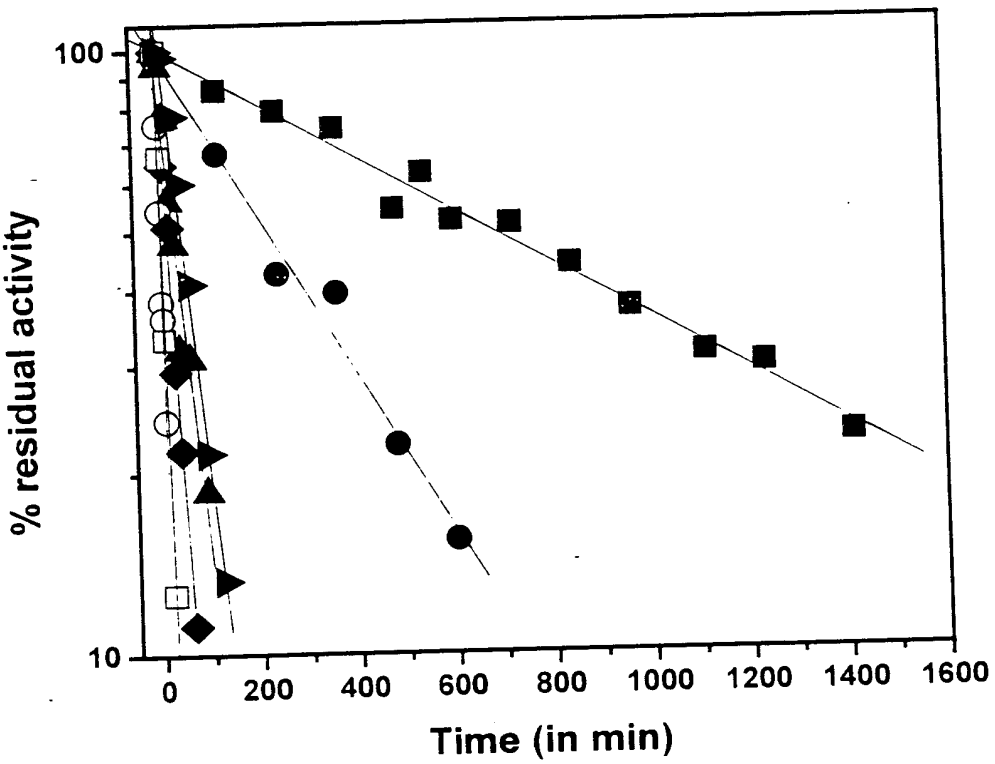
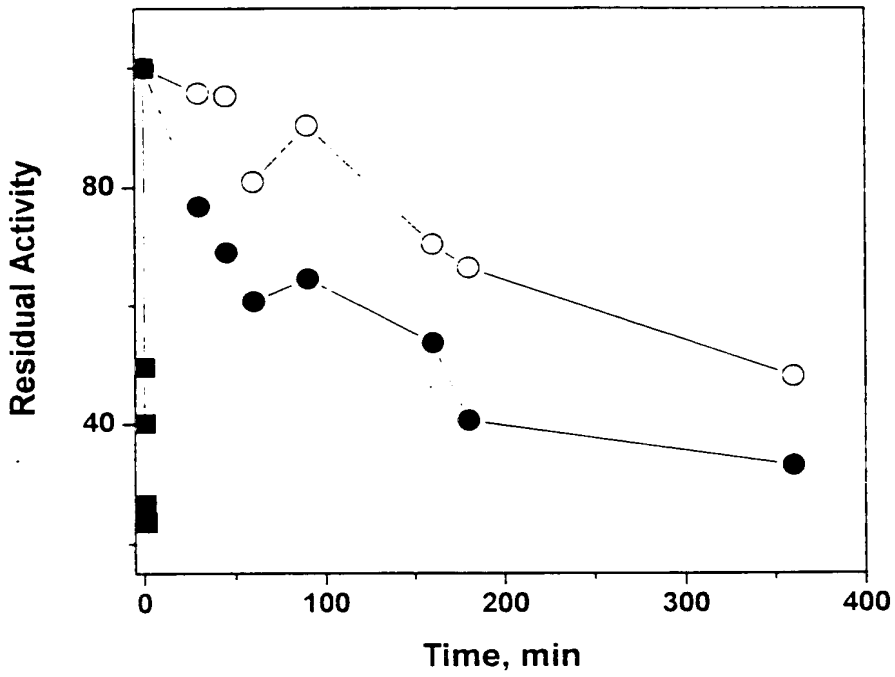


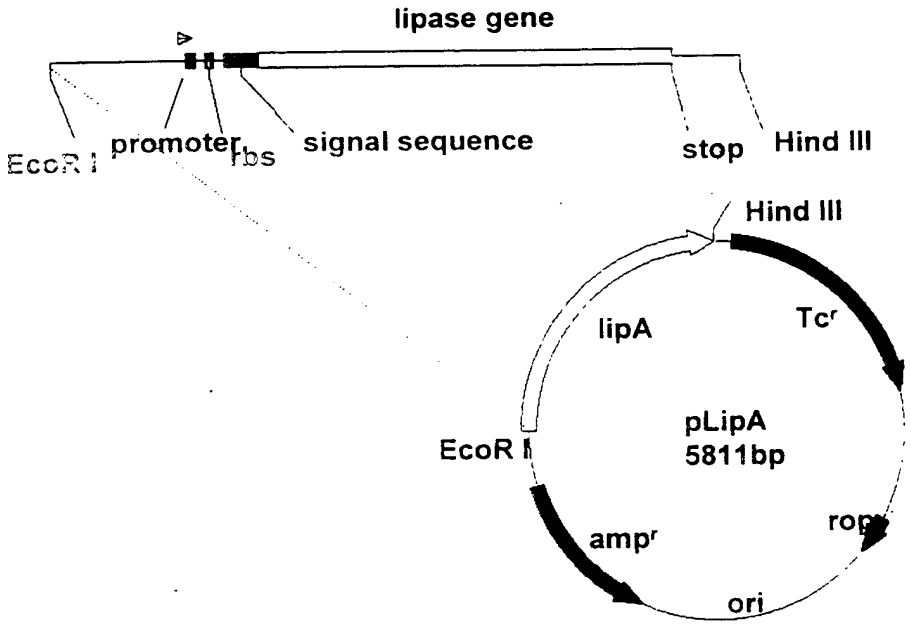
Fig.4



Residual activity of two mutants and a wild type using olive oil as the substrate.

The method of measurement of activity was by pH stat.

Fig.6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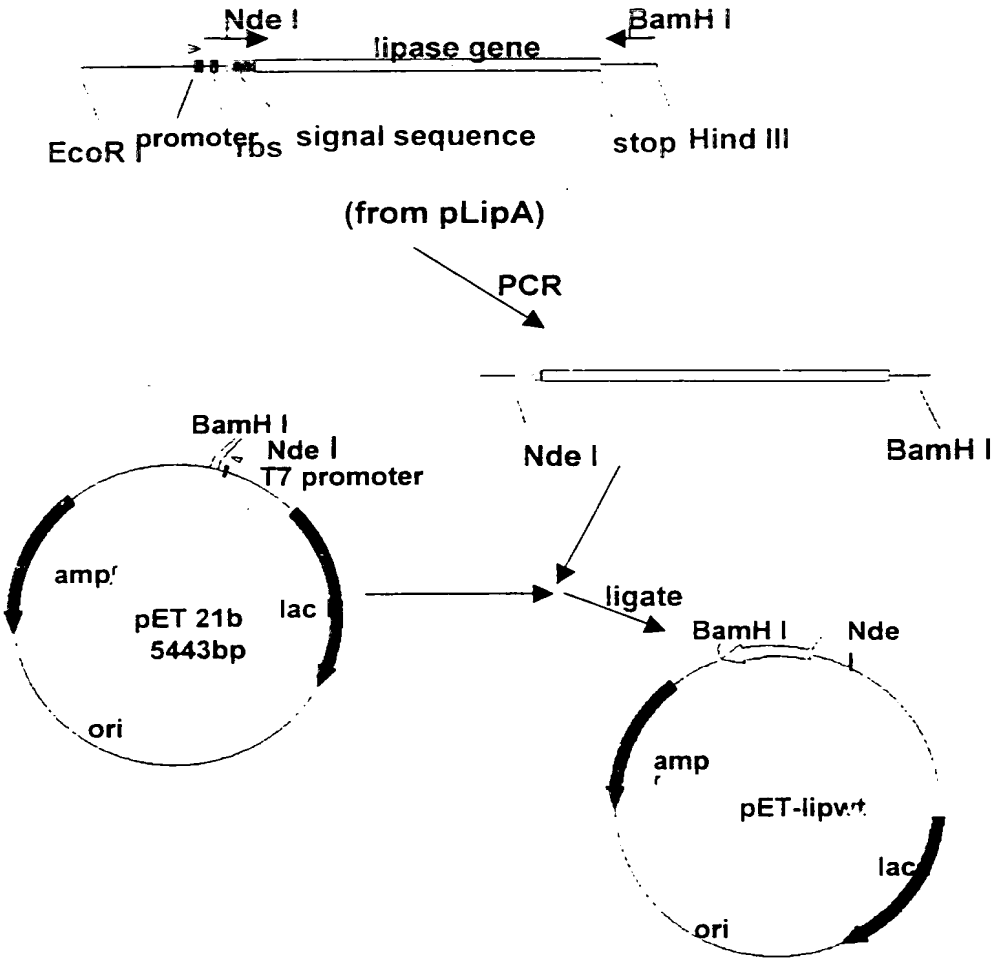
EcoR I
promoter
rbs
signal sequence
stop
Hind III
Hind III
lipA
Tcr
pLipA
5811bp
EcoR I
ampr
ori

Fig.67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BamH I
lipase gene
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promoter
rbs
signal sequence
stop Hind III
(from pLipA)
PCR
BamH I
Nde I
T7 promoter
Nde I
BamH I
amp
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lac
ori
ligate
BamH I
Nde
I
amp
pET-lipwt
lac
ori

Fig.8

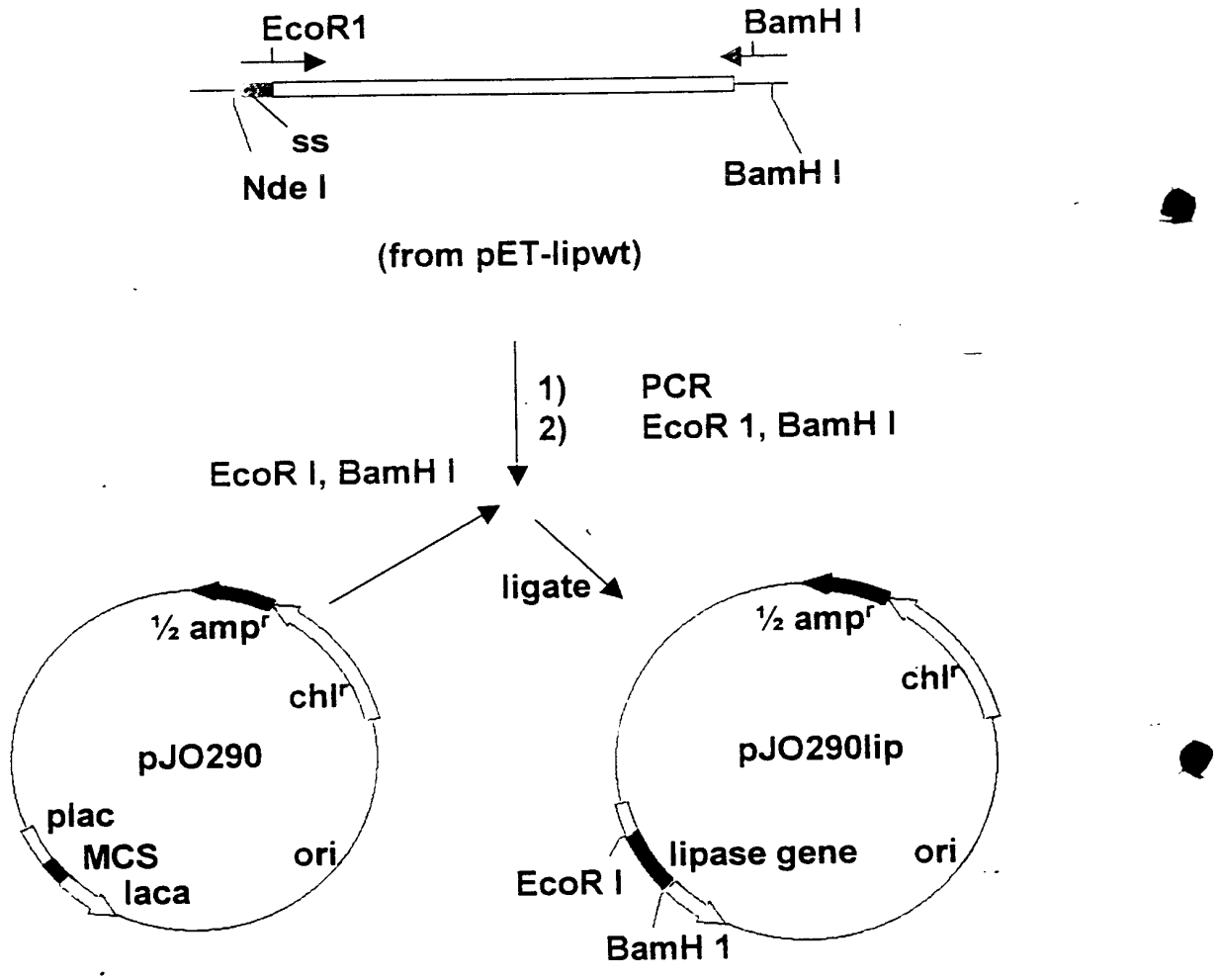
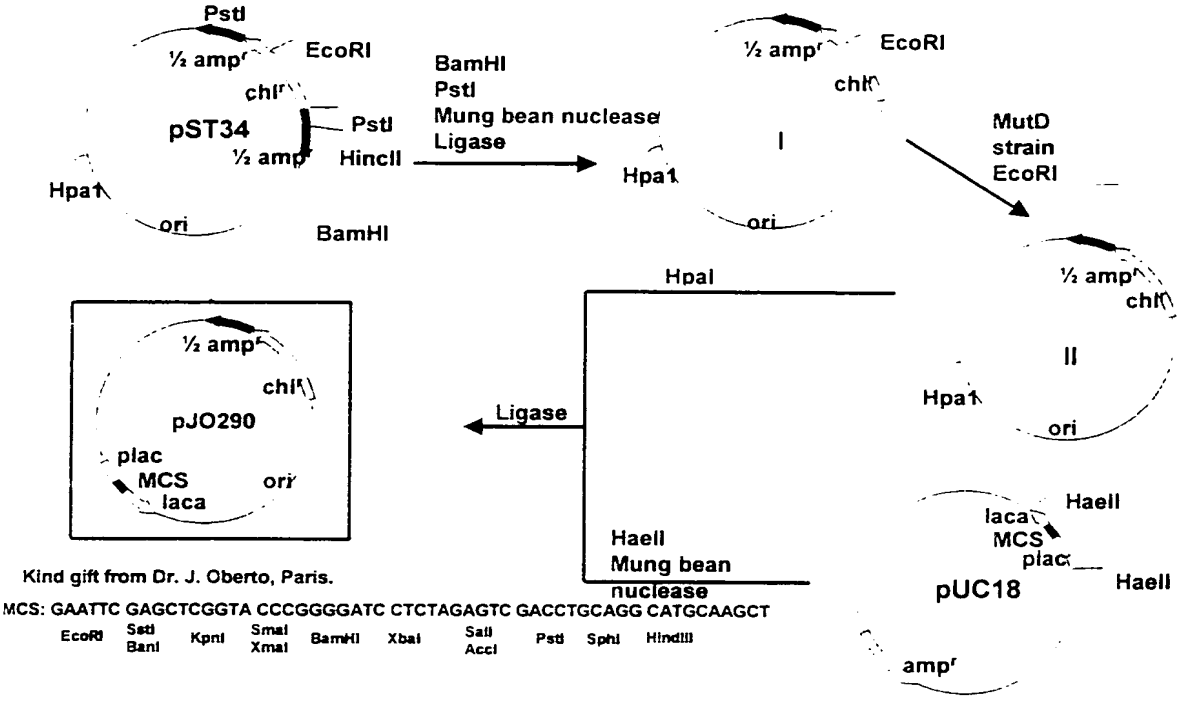

EcoR1
BamH I
ss
Nde I
BamH I
(from pET-lipwt)
1) PCR
2) EcoR 1, BamH I
EcoR I, BamH I
ligate
½ ampr
chlr
pJO290
plac
MCS
laca
ori
½ ampr
chlr
pJO290lip
EcoR I
lipase gene
ori
BamH 1

Fig.9



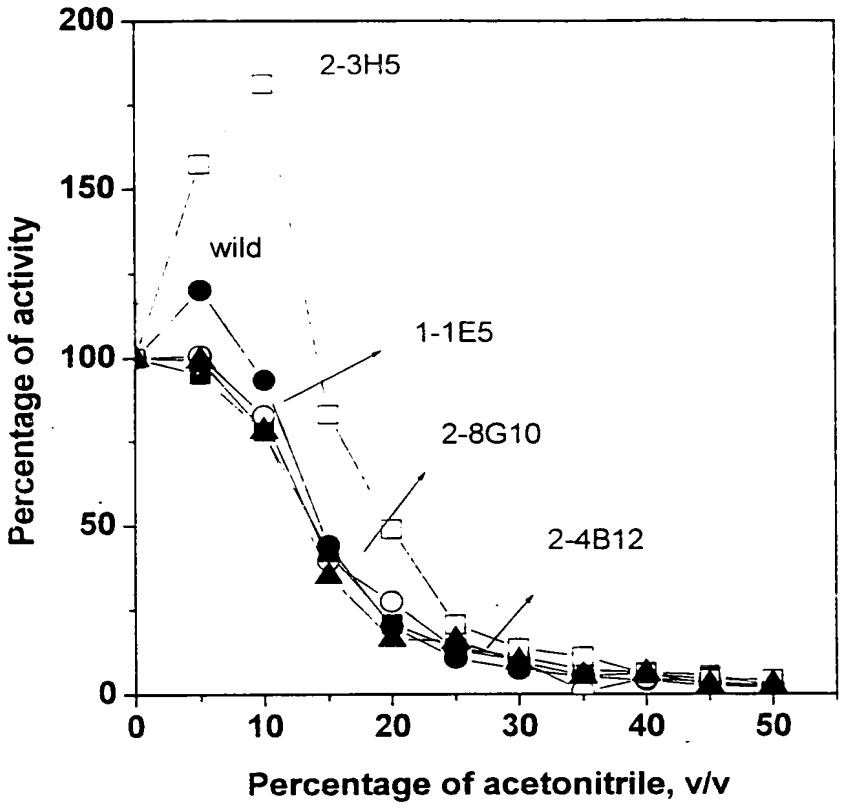
Accordingly

Fig.10

Kinetic parameters for the wild-type lipase and the thermostable mutants

Clone	K_m, mM	k_{cat} X 10^{-2}, min^{-1}	k_{cat}/K_m X 10^{-2}, $mM^{-1}.min^{-1}$	T ½, min (55°C, pH 7.0)
wt	0.97	5.2	5.4	2.5
1-1E5	1.03	5.0	4.85	25.4
2-8G10	0.69	5.5	8.0	18.9
2-3H5	0.63	6.1	9.7	7.0
3B1	1.22	6.8	5.6	228.0
3B2	1.45	7.5	5.2	46.1
4B1	1.96	8.1	4.1	677.0

Fig.11


200
150
100
50
0
Percentage of activity
0
10
20
30
40
50
Percentage of acetonitrile, v/v
2-3H5
wild
1-1E5
2-8G10
2-4B12

Total number of sequences:

Protein sequences 12
Gene Sequences 12

All gene sequences are of 552 base pairs of linear single stranded DNA.
All protein sequences are of 181 amino acids in length and have enzymatic properties. It should be noted that the sequences presented here are identical to the published lip A sequence except for the first amino acid , methinine present in our sequence. The sequence purified and crysallized in literature does not have the N-terminal methionine.

Protein Seq ID #1

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GGHIGLLYSSQVNSLIKEGLNGGGQNTN

Gene Seq ID#1

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Protein Seq ID #2 1-1E5 (N166Y) (N167Y)

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Protein Seq ID # 3 2-8G10 (N166Y; A132D; a68V) (N167Y; A133D; a69V)

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Gene Seq ID # 3
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Protein Seq ID # 4 2-3H5 (N166Y; L114P; A68T) (N167Y; L115P; A69T)

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Gene Seq ID# 4

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ccagaatacg aattaatga

Protein Seq ID # 5 3B1 (N166Y; A132D) (N167Y; A133D)

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Gene Seq ID # 5
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aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg
tgcgaaaaaagtggatattg tcgctcacag catgggggge gcgaacacac tttactacat
aaaaaatctggacggcggaa ataaagttgc aaacgtcgtg acggttggcg gcgcgaaccg
tttgacgacaggcaaggcgc ttccgggaac agatccaaat caaaagattt tatacacatc catttacagc
agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg
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ccagaatacg aattaatga

Protein Seq ID # 6 4B1 (N166Y; A132D; L114P) (N167Y; A133D; L115P)

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GGHIGLLYSSQVYSLIKEGLNGGGQNTN

Gene Seq ID # 6
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tttgacgacaggcaaggcgcccc ccggggaac agatccaaat caaaagattt tatacacatc catttacagc
agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg
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Protein ID # 7 2-4B12 (N166Y; R147S) (N167Y; R148S)

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GGHIGLLYSSQVYSLIKEGLNGGGQNTN

Gene Seq ID # 7
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agtgccgata tgattgtcat gaattactta tcaagattag atggtgctagtaacgttcaaatccatggcg
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Protein Seq ID # 8
3B2 (N166Y; L114P) (N167Y; L115P)

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VTLGGANRLTTGKAPPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQIH
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Gene Seq ID # 8
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agtgccgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg
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Protien Seq ID # 9

2-3E8 (N166Y; V71A) (N167Y; V72A)

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Gene Seq ID # 9
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agtgccgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg
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Protein Seq ID #10 2-3E9 (N166Y; D144V) (N167Y; D145V)

MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
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Gene Seq ID #10
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Protein Seq ID # 11 3-2B5 (N166Y; A132D; A68V; N120D) (N167Y; A133D; A69V; N121D)

MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
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GGHIGLLYSSQVYSLIKEGLNGGGQNTN

Gene Seq ID # 11

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aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg
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agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg
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Protein Seq ID # 12quadruple mutant (N166Y; A132D; L114P; N121D) (N167Y; A133D; L115P; N121D)

MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKAPPGTDPDQKILYTSIYSSDDMIVMNYLSRLDGARNVQIH
GGHIGLLYSSQVYSLIKEGLNGGGQNTN

Gene Seq ID #12

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ttggacacat cggccttctg tacagcagcc aagtctacag cctgattaaagaagggctga acggcggggg
ccagaatacg aattaatga

Dated the 20th day of January 2003

FORM 2

THE PATENTS ACT –1970

COMPLETE SPECIFICATION

(See Section 10)

STABLE GENE VARIANTS OF LIPASES

COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH

Rafi Marg, New Delhi –110 001, India, an Indian Registered Body

Incorporated under the Registration of Societies Act (XXI of 1860)

The following specification particularly describes the nature of the nvention and the manner in which it is to be performed.

STABLE GENE VARIANTS OF LIPASES

TECHNICAL FIELD

The present invention relates to the generation and production of novel thermostable, organic solvent stable and pH tolerant lipase gene variants. The invention also relates to methods of selecting lipase variants for high temperatures and for their purification.

BACKGROUND AND PRIOR ARTS

Enzymes are the workhorses of a cell that affect virtually every biological process that characterizes a living organism. They catalyze chemical reactions with remarkable specificity and rate enhancements. The awesome catalytic power and versatility of enzymes has long been recognized and enzymes have proved to be very useful outside the living system as well. Enzymes today have widespread application in industry and are seen as environment friendly alternatives to chemical reagents because enzymatic reactions require milder conditions and tend to be cleaner with lesser byproduct and waste generation. Enzymes are being used in numerous new applications in the food, feed, agriculture, paper, leather, and textiles industries, resulting in significant cost reductions and environment-friendly operations.

Enzymes have evolved to function best under the physiological conditions of the parent organism. *In vitro* applications often call upon enzymes to work under non-physiological conditions or to perform functions they have not evolved for. For example, enzymes may have to catalyze reactions involving novel substrates; they may have to work under extreme conditions of salt, temperature, pH etc., or in the presence of potentially inhibiting or denaturing chemicals. Such applications have brought to light the severe disabilities of enzymes to function as industrial catalysts. In order to extract optimum performance from enzymes in the test-tube and in industrial reactors, these biocatalysts need to be tailored to suit specific applications.

The commercial success of these enzymes can be attributed to their ease of use. In addition to functioning at high temperatures, thermostable enzymes generally posses an increased shelf life which markedly improves handling conditions. If enzymes are to play a significant role in large scale processing of chemicals, they must be able to endure the harsh conditions associated with these processes. Thermostable enzymes are easier to handle, last longer, and given the proper immobilization support should be reusable for multiple applications

In obtaining thermostable enzymes the conventional approach is to screen the microbial collections collected from extremephillic environments (Karsheroff and Ladenstein, 2001). The promising candidate enzymes are further investigated for suitability for a specific process. For example, for applications requiring thermostable or salt stable enzymes, enzymes from thermophilic or halophilic organisms were used, respectively. However, such an approach

severely restricted the use of enzymes because enzymes for all applications cannot be found in nature. There may not be a natural enzyme for many kinds of transformations. Moreover, enzyme usage is often restricted by undesirable properties of enzymes like product inhibition, low stability etc. Very often an enzyme is required to have a combination of several properties that may be impossible to find in a natural enzyme.

Another approach to obtain thermostable enzymes is based on the current knowledge on the protein structures (crystal structures) of homologous enzymes from mesophiles and thermophiles (Kumar *et al*, 2000; Lehmann *et al*, 1998). Such comparisons yielded information on the probable interactions that enhance thermostability. Using such information efforts were made to incorporate these changes in mesophillic enzymes to improve their thermostability. Such approaches have not been very successful since interactions that improve thermostability in a protein are many and each protein acquires, over evolutionary times, those interactions that are best suited for its sequence and the mileu in which it functions. Though structural determinants of protein stability have been objects of numerous studies on model proteins, no universal stabilization mechanism has yet been found (Jaenicke and Bohm, 1998). The most obvious conclusion that can be drawn from the literature is that different proteins have adapted to different thermal environments by a variety of evolutionary devices. The lack of understanding of the structural features leading to protein thermostability has been partly due to a scarcity of data because experimental studies comparing homologous proteins from psychrophilic, mesophilic and thermophilic organisms have been limited to only a few proteins. Moreover, inability to form definite rules for improving protein thermostability is due to the large number and complexity of possible contributing factors (Jaenicke and Bohm, 1998; Vogt *et al.*1997a; Vogt *et al.* 1997b; Ladenstein and Antranikain, 1998). Based on comparisons between mesophiles and thermophiles, the main mechanisms responsible for increased thermostability have been identified as increase in the number of hydrogen bonds and salt bridges, increased core hydrophobicity, better packing efficiency, α-helix and loop stabilization and resistance to covalent destruction. Often it becomes difficult to delineate protein interactions that contribute to thermostability from other selection pressures such as salt, pH etc.

Other strategies adapted to increase the thermostability was based on the observation that immobilized enzymes acquire thermostability to some extent (Reetz *et al.*, 1995). Hence, several solid supports were tried to immobilize proteins. And also recent observations made with enzymes in organic solvents indicated that in organic solvents enzymes acquire thermostability (Plou and Ballesteros, 1999). The advent of recombinant DNA techniques has greatly facilitated protein engineering by allowing facile mutagenesis and production of proteins.

The term protein thermostability refers to the preservation of the unique chemical and spatial

structure of a polypeptide chain under extremes of temperature conditions (Jaenicke and Bohm, 1998). In general, the higher the temperature to which the enzyme is exposed, the shorter the half-life of the enzyme (i.e., the shorter the enzyme retains its activity). Similarly, the greater levels of organic solvent to which said enzymes are exposed, the shorter the half-life of the enzyme. The phrase "catalytic activity" or simply "activity," means an increase in the k.sub.cat or a decrease in the K.sub.M for a given substrate, reflected in an increase in the k.sub.catt /K.sub.M ratio.The structural basis of protein thermostability has been an actively pursued area of research for at least two decades (Argos *et al.*, 1979). However, enzymes lifted out of the context of living organisms do not always function as well as they do in their natural milieu. For example, they have optimum activity at the physiological temperature of the organism and tend to denature at higher temperatures leading to drop in activity. Thermostable enzymes are important as they can be used at high temperatures and harsher conditions required in industrial contexts. Also they generally have higher storage stabilities and bring down costs by obliviating the need for low temperature storage and decreasing the loss due to denaturation on storage and handling. Moreover reactions carried out at higher temperatures generally proceed at higher rates further bringing down operation times.

In view of the environmental safety reasons, there is a constant pressure to reduce the use of environmentally polluting processes in industry. Enzymes are increasingly used to replace chemical processes in leather, food, and pharmaceutical industries. Comparison of protein structures from extremeophiles demonstrated that protein structural plasticity is enormous and is resident in the primary structure. This lent considerable support to strategies that alter the primary structure of the proteins at the genetic level and screen for the variants with special properties such as thermostability. The tremendous success in handling the genes and developing protocols to alter it at will, has allowed to evolve proteins with special functions. The strategy relies in generating variation in gene sequences by molecular biology methods and screening the variants by expressing them and screening the mutant population (Arnold, 1999; Stemmer, 1994; Ostermeier *et al.*, 1999). The screening protocols are based on the property of interest, e.g., activity at high temperature or activity in the presence of organic solvents. The present invention encompasses methods for generating variation in gene sequences, protocols for screening the enzymes with higher thermostability and also protocols for sequencing and expression.

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of ester bonds in triacylglycerols and often also exhibit phospholipase, cutinase and amidase activities (Woolley and Petersen, 1994). They are used for the production of detergents, pharmaceuticals, perfumes, flavour enhancers and texturising agents in consmetic

products. Lipases are crucial for the production of a wide variety of foods, especially for products from milk, fat and oil. Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface and do not hydrolyze dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases (Jaeger *et al.*, 1999). Many lipases are active in organic solvents where they catalyze a number of useful reactions including esterification transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides and other chemicals. An increasing number of lipases with suitable properties are becoming available and efforts are underway to commercialize biotransformation and syntheses based on lipases (Schmid and Verger, 1998). Enzyme sales for use in washing powders still remain the single biggest market for industrial enzymes. The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year Because of their ability to hydrolyzes fats, lipases find a major use as additives in industrial laundry and household detergents. Detergent lipases are especially selected to meet the following requirements: (1) a low substrate specificity, i.e., an ability to hydrolyze fats of various compositions; (2) ability to withstand relatively harsh washing conditions (pH 10–11, –60 _C); (3) ability to withstand damaging surfactants and enzymes [e.g., linear alkyl benzene sulfonates (LAS) and proteases], which are important ingredients of many detergent formulations. Lipases with the desired properties are obtained through a combination of continuous screening (Jaeger and Reez, 1998; Wang *et al.*; Rubin and Dennis, 1997) and protein engineering (Kazlauskas and Bornscheuer, 1998). In 1994, Novo Nordisk introduced the first commercial recombinant lipase 'Lipolase,' which originated from the fungus Thermomyces lanuginosus and was expressed in Aspergillus oryzae. In 1995, two bacterial lipases were introduced—'Lumafast' from Pseudomonas mendocina and 'Lipomax' from P. alcaligenes—by Genencor International (Jaeger *et al.*, 1999). According to a report an alkaline lipase, produced by P. alcaligenes M-1, which was well suited to removing fatty stains under conditions of a modern machine wash. The patent literature contains examples of many microbial lipases that are said to be suitable for use in detergents (Gerritse *et al.*, 1998).

Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomyces. Bacillus

subtilis is a Gram-positive, aerobic, spore-forming bacterium that has generated substantial commercial interest because of its highly efficient protein secretion system Though extracellular lipolytic activity of B.subtlis was observed as early as in 1979 (Bycroft and Byng, 1992), molecular research started in 1992 when a lipase gene, lipA, was cloned and sequenced (Dartois *et al.*, 1992). Subsequently the lipase was overexpressed, purified and characterized (Leuisse *et al.*, 1993). Later, a second gene, lipB, was found that is 68% identical with lipA at the nucleic acid level (Eggert *et al.*, 2000). This gene has been cloned and the protein overexpressed, purified and characterized.

The *Bacillus subtilis* lipase with a molecular weight of 19,348 Da is one of the smallest lipases known. It is one of the few lipases that do not show the interfacial activation in the presence of oil-water interfaces. LipA is very tolerant to basic pH and has its optimum activity at pH10. It hydrolyses the sn-1 and sn-3 glycerol esters with both short and long chain fatty acids, showing optimum activity with C8 fatty acid chains.

Bacterial lipases are classified into eight families according to their sequence similarities, conserved sequence motifs and biological properties (Arpigny and Jaeger, 1999). The true lipases are classified in family I which contains six subfamilies. Bacillus lipases have been placed in subfamilies 4 and 5. In these two subfamilies alanine replaces the first glycine residue in the conserved G-X-S-X-G pentapeptide around the active site serine residue. Subfamily 4 consists of only three members, LipA and LipB from B.subtilis and a lipase from *Bacillus pumilis*, which share 74-77% sequence identity. These are the smallest lipases known and show very little sequence similarity (~ 15%) with the other, much larger, Bacillus lipases that constitute subfamily 5.

The crystal structure of the B.subtilis lipase LipA reveals a globular protein with dimensions of 35 X 36 X 42 (Pouderoyen *et al.*, 2001). The structure shows a compact domain that consists of six β- strands in a parallel β- sheet, surrounded by α- helices. There are two α-helices on one side of the β- sheet and three on the other side. The fold of the B.subtilis lipase resembles that of the core of the α/β hydrolase fold enzymes. The B.subtilis lipase lacks the first two strands of the canonical α/β hydrolase fold and the helix αD is replaced by a small 310 helix. The helix αE is exceptionally small, with only one helical turn, and several α-helices start or terminate with 310 helical turns. Due to these structural features, its small size and absence of a lid domain, the B.subtilis lipase is considered a minimal α/β hydrolase fold enzyme.

OBJECTS OF THE INVENTION

The main object of the present invention relates to novel thermostable, organic solvent stable and pH tolerant lipase gene variants.

Another object of the present invention relates to an expression system comprising of novel

thermostable, organic solvent stable and pH tolerant lipase gene variants.

Yet another object of present invention relates to a method of preparing an expression system, said system comprising of novel thermostable, organic solvent stable and pH tolerant lipase gene variants.

One more object of the present invention relates to the gene variants wherein the gene have inherent ability to withstand high pH in the range of 10 to 11; ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof.

Another object of the present invention relates to the gene variants wherein gene variants are useful as stain remover in household detergents and laundry industry.

Yet another object of the present invention relates to the gene variants wherein the gene variants have extremely high specific activity.

BRIEF DESCRIPTION OF ACCOMPANYING FIGURES / DRAWINGS:

Fig.1 : The lipA gene pBR 322. The lipA gene containing the entire lipase gene sequence along with the signal sequence, promoter and the ribsome binding sequence was shown.

Fig.2: Subcloning of lipA with the signal sequence into pET 21b. The lip A gene along with the signal sequence was amplified using the For1 and Rev1 primers and then was inserted into the pET21b resulting in pEt-lipwt. The For1 and Rev1 primers were designed to introduce Nde 1 and Bam H1 sites into the LipA gene.

Fig.3: Subcloning of lipA without signal sequence into pJO290. The lipA gene present in the pET-lipwt was amplified using the PrEcoR1 and PRBam H1 primers designed specifically to introduce EcoR1 and Bam H1 sites into the amplified products. The amplified product was cut using EcoR1 and Bam H1 and then inserted into pJO290 vector which was similarly cut.

Fig.4: SDS-PAGE profiles of the purified proteins. All the lipases were purified by the procedures given in examples.: Low Molecular weight marker, Lane 1; wild-type lipase, Lane 2; Gene sequence 2, Lane 3; Gene sequence 3, Lane 4; Gene sequence 4, Lane 5; Gene sequence 5, Lane 6; Gene sequence 6, Lane 7.

Fig.5: Hydrolysis of a triglyceride catalyzed by a lipase. The schematic of the action of lipase on triglycerides is given. Lipase acts on the ester bonds and produces free fatty acids and glycerol.

Fig. 6: Residual activity of various mutants and the wild type at a various times on

exposure to a temperature of 55 C. The substrate used is PNPA. Wild type and the mutant enzymes were incubated at 55 C fro various lengths of time and the activity was estimated on cooling the enzyme on ice for few minutes. The activity was estimated at room temperature. The activity was expressed as the rate of hydrolysis of PNPA. Hydrolysis of PNPA was monitored as increase in absortion at 410 nm in a spectrometer.

Fig.7: Residual activity of the wild type and the mutants exposed to 55 C was provided as table. The protein was incubated for specified time and the activity was estimated after cooling the enzyme on ice and then assaying for the activity at room temperature .

Fig.8: Residual activity of various mutants and the wild type at various times on exposure to temperature of 50 C. The substrate used is olive oil. Substrate olive oil was emulsified using gum Arabic. The rate of hydrolysis of olive oil was monitored in a pH stat (Metrohm 718 pH Titrino) as the rate of addition of 0.1 m sodium hydroxide per min. Hydrolysis of olive oil decrease the pH which was neutralized by sodium hydroxide.

Fig.9: Kinetic parameters and half life of stability at 55 C of wild type and mutant lipase. Thermostability of the wild type and the mutants was represented as the slope of the plot obtained by plotting residual activity vs. time. Lower slope indicates longer half life, hence increase in thermostability. Enzyme constants were measured by conducting detailed activity vs. substrate concentration with each of the mutants. These plots were used to estimate the apparent equilibrium constants and the V max i.e. velocity at the infinite concentration of the substrate. Km / V max represents the catalytic potential of the enzyme, which is a useful parameter to compare the catalytic potential between enzymes.

Fig.10: Activity of lipase and its mutants in the presence of acetonitrile at various concentrations in water. Wild type and mutant enzymes were incubated in a medium containing various amounts of acetonitrile. The remaining activity at a fixed incubation time of 30 min was estimated using PNPA as substrate.

Fig.11: Residual activity of wild type lipase and the mutants were assayed after incubating the protein in 20 % acetonitrile for 30 min.

Fig.12: Wild type lipase was incubated in the presence of various amounts of three different detergents for 30 min and the activity was assayed after 30 min. The three

detergents were neutral detergent Triton X-100, anionic detergent , sodium dodecyl sulphate and cationic detergent , cetyl trimethylammonium bromide.

SUMMARY OF THE INVENTION

The present invention relates to novel gene variants of lipase enzyme as developed through site directed muatgenesis. These gene variants are highly thermostable, resistant against strong organic solvents and can tolerate high pH. The thermostability of the developed gene variants was as high 200 fold in the temperature range of 50 to 90°. The developed gene variants due to

high thermostability, specific activity and tolerance due to high pH have application in household detergents and laundry industry.

DESCRIPTION OF THE INVENTION:

In the present invention a method of directed evolution were applied to the lipase gene (Gene Seq ID No.1) to isolate protein variants of the original sequence which possess increased thermostable properties. The methodology relies initially on the ability to create random variations in the original gene sequence and express the corresponding proteins in the bacteria, E.coli. The produced variants of the original sequence would have altered sequence, hence altered properties. The variants, at a proteins level , would be tested for their thermostability and those sequences which demonstrate improved thermostability would be subjected to the next round of random mutagenesis and screening. Thus by sequential accumulation of the mutants and subsequent pooling of the mutations the thermostability of lipase was improved by 200-fold at high temperature. High temperature range includes temperature ranges from 50-90 C.

The method according to the present invention includes four steps in generating the variant lipases and their characterization to obtain thermostable lipases. In the first of the methods variation in the primary sequence of the lipase gene were generated by error prone PCR methods. The adapted protocols are similar to several published protocols. In addition many of the different protocols such as random priming, ITCHY etc could also be applied for generation of variance in the gene sequence (Lutz and Benkovic, 2000; Shao *et al.*, 1998). In the second step of the invention, the mutant sequences are cloned into an expression vector and the protein expressed in the culture lysates. The expressed proteins are screened for their ability to withstand higher temperature was tested in a large population using medium-throughput methods. In the third step of the invention the promising variants were pooled by a family shuffling procedures according to the method of Stemmer (1994) and further tested for thermostability. In addition to the shuffling procedures selective changes in the primary sequence were also incorporated by standard molecular biology procedures. In the fourth step of the invention the positive sequences were over-expressed in large cultures and the proteins

are purified by the published procedures according to Dartois *et al* (1992). The purified proteins were tested for their thermostability.

The screening procedures of the lipase variants for increased thermostability involve ability to hydrolyse chromogenic substrate esters based on p-nitrophenyl group. Natural substrates of lipase are triglycerides, which are not convenient to design a simple medium trough put assays wherein the source of enzyme is over-expressed lipase in a cell lysate (Besson *et al.*, 2000). P-nitrophenyl esters of fatty acids are convenient and the activity of lipase represents the activity of lipase on triglycerides. Long chain esters of p-nitrophenyl, especially p-nitrophenyl oleate,

are well suited for this purpose. Detergent solubilized PNPO demonstrates negligible back ground hydrolysis and well suited for lipase present in cell lysate. The strong yellow color of very high extinction coefficient of hydrolysis product p-nitrophenyl can be estimated conveniently in a 96-well plates. Along with p-nitrophenyl esters, many other fluorogenic or chromogenic esters of fatty acids could be used for this purpose.

As employed herein the term thermostability refers to the property of the enzymes, which retain their activity subsequent to exposure to higher temperatures. Enzymes lose their tertiary conformation on exposure to higher temperature due to the increased movement of the structural elements, which perturbs the functional structure of the protein. Typically proteins lose their activity at higher temperatures with time. The rate of this loss in activity, reflects in half life i.e., time required to lose half of the initial activity, is a convenient parameter to compare the thermostability of the protein (Jaenicke and Bohm, 1998). Activity, as defined here, corresponds to the catalytic acitivity represented by the term kcat/Km, where kcat is the rate of the product formation and Km is the apparent affinity constant of the substrate to the enzyme. Retaining the functional structure at elevated temperatures resides in the ability to form interactions within the protein that withstand high temperatures. The range of the temperature that is relevant for the present invention ranges from 35 to 90 C.

The naturally occurring lipase from *Bacillus lipase* has the amino acid sequence of 1-181 as given in the SEQ ID No.1. It was discovered that the amino acid substitutions at positions 68, 71, 114, 120, 132, 144, 147 and 166 were found to be important for the thermostability of the lipase. In accordance with the present investigation, it was further discovered that the substitutions at positions 114, 132 and 166 are suited for increasing the stability of the proteins. Any of the innumerable combinations of substitutions possible at each of these positions with the other 19 amino acids would be favourable for the thermostability.

The specific substitutions of relevance for thermostability in lipase are given below.

From	To	Position
N	V	166

A	D	132
A	V	68
L	P	114
R	S	147
V	A	144
N	D	120

Accordingly, the main embodiment of the present invention relates to the novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants having SEQ ID No. 2 of molecular wt 19443, SEQ ID No. 3 of molecular wt 19515 SEQ ID No. 4 of molecular wt 19456.9, SEQ ID No.5 of molecular wt.19487 and and SEQ ID No.6 of molecular wt. 19470.9.

Another embodiment of the present invention relates to an expression system for novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants said expression system comprising of having SEQ ID No. 2 of molecular wt 19443, SEQ ID No. 3 of molecular wt 19515, SEQ ID No. 4 of molecular wt 19456.9, SEQ ID No.5 of molecular wt. 19487 and SEQ ID No.6 of molecular wt 19470.9 present in the vector pJO290.

Still another embodiment of the present invention relates to the a method of preparing an expression system of novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants having SEQ ID No. 2 of molecular wt 19443, SEQ ID No. 3 of molecular wt 19515, SEQ ID No. 4 of molecular wt 19456.9, SEQ ID No.5 of molecular wt. 19487 and SEQ ID No.6 of molecular wt 19470.9, said method comprising the steps of :

(a) isolating and purifying lipase gene from *Bacillus subtilis*,

(b) cloning lipase gene isolated in step (a) in vector pJO290,

(c) generating gene variants from lipase gene isolated in step (a) by random mutagensis and site-directed mutagenesis using forward primer JOF having SEQ ID No.13 and reverse primer JOR having SEQ ID No. 14,

(d) cloning the gene variants obtained in step (c) in plamsid vector pJO290, and

(e) ligating the cloned gene variants of step (d) in *E.coli* JM109.

Another embodiment of the present invention relates to the gene variants wherein gene variants are thermostable in the temperature range of about 45 to 95°C.

One more embodiment of the present invention relates to the gene variants wherein said gene variants are highly thermostable at the temperature in the range of about 55 to 90°C.

Still another embodiment of the present invention relates to the $T_{1/2}$ value of novel gene variants wherein $T_{1/2}$ value is in the range of 6 to 685.

Yet another embodiment of the present invention relates to the $T_{1/2}$ value of novel gene variants as wherein $T_{1/2}$ value is in the range of 7 to 677.

Another embodiment of the present invention relates to Km value of gene variants wherein Km value is in the range of 0.50 to 2.5 mM.

Still another embodiment of the present invention relates to the Km value of novel gene variants wherein Km value is in the range of 0.63 to 1.96 mM.

One more embodiment of the present invention relates to k_{cat} value of the novel gene variants wherein k_{cat} value is in the range of 4.5×10^{-2} to 8.5×10^{-2} min^{-1}.

Still another embodiment of the present invention relates to the k_{cat} value of novel gene variants wherein k_{cat} value is in the range of 5×10^{-2} to 8.1×10^{-2} min^{-1}.

Yet another embodiment of the present invention relates to the k_{cat}/K_m value of novel gene variants wherein k_{cat}/K_m value is in the range of 4×10^{-2} to 10×10^{-2} min^{-1}.

Another embodiment of the present invention relates to the k_{cat}/K_m value of novel gene variants wherein k_{cat}/K_m value is in the range of 4.1×10^{-2} to 9.7×10^{-2} min^{-1}.

One more embodiment of the present invention relates to the resistance of novel gene in organic solvents wherein organic solvents are selected from group of acetonitrile, isopropanol, dimethyl sulfoxide and dimethyl formide.

Still another embodiment of the present invention relates to the organic solvent used wherein organic solvent used is acetonitrile.

One more embodiment of the present invention relates to the residual activity of the gene variants wherein gene variants have residual activity in the range of 25 to 100 % in presence of acetonitrile.

Another embodiment of the present invention relates to the residual activity of the gene variants wherein gene variants have residual activity in the range of 28.7 to 85.5 % in presence of acetonitrile.

Still another embodiment of the present invention relates to the gene variants wherein the gene variants have inherent ability to withstand high pH in the range of 9 to 13;

ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof

One more embodiment of the present invention relates to the to the gene variants wherein the gene variants have inherent ability to withstand high pH in the range of 10 to 11; ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof

The following examples are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention.

EXAMPLES:

EXAMPLES:

Example 1:

Purification of lipase from Bacillus subtilis

Purification of the lipase was performed from E.coli cells expressing the lipase in an appropriate vector. The purification essentially involves passing the cell lysate in phenyl-sepharose column followed by a Mono-S column. Lipase is an aggregated prone protein, care especially keeping protein concentration below 5mg/ml, was taken to avoid aggregation of the protein. The purification of the lipase was carried out essentially as described earlier[32] with minor modifications. Lipase from culture filterates of Bacillus strain or from the E.coli lysates was processed similarly. For purification of the wild type and mutant proteins from E.coli the lipA gene or the mutant genes are cloned into pET 21b.). For this, the gene corresponding to the full length, mature protein was amplified with primers PrNde I (forward primer) (5'-CCATGATTACGCATATGGCTGAACACAA-3') and JOF. The forward primer had an engineered Nde I site. The forward primer also introduced a start codon at the start of the lipase gene in the form of the ATG sequence that is part of the Nde I recognition sequence. This would introduce a methionine in the N-terminus of the mature protein, expressed in *E.coli*, just before the N-terminal alanine that occurs in the protein purified from the culture supernatant of *B.subtilis*. The wild type protein as well as the mutants were amplified, digested with Nde I and BamH I and ligated with pET-21b digested with Nde I and BamH I. The ligation mix was transformed into *E.coli* DH5α and the positives were selected by plasmid minipreps and restriction digestions (Fig.1 and Fig.2).

Protein was purified from *E.coli* BL21 (DE3) cells. Cells containing the appropriate plasmid were grown till mid-log phase before inducing with 0.5 mM IPTG. Cells were harvested 2.5 hours after induction by centrifuging at 15,000 rpm at 4 °C for 20 min. The pellet was washed with STE and resuspended in 1X TE containing 0.3 mg/ml lysozyme. The suspension was incubated on ice for 30 min before lysing the cells by sonication. Sonication was carried out by keeping the cells on ice. Short pulses of half-minute duration were applied and 1 min cooling time was allowed between pulses. The sonicated cells were centrifuged at 20,000 rpm at 4 °C. The supernatant was loaded on a phenyl sepharose column. The remaining steps were done as described in chapter 2. The purified proteins were stored in –70 °C till further use.

B.subtilis BCL1051 was grown aerobically for 16-18 hrs at 37 °C in 2l Erlenmeyer flasks, each containing 500 ml of medium of the following composition: 2.4 % yeast extract, 1.2 % tryptone, 0.4 % gum Arabic, 0.4 % glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4, 50 mg/ml kanamycin sulfate. The culture medium was inoculated at 1 % from 10 ml precultures. After harvesting the cells by centrifugation at 6000 rpm for 30 min, the culture supernatant was pumped at a flow rate of 30 ml/hr onto a Phenyl Sepharose Fast Flow High sub column (Pharmacia) (20 ml column volume per 1l culture) equilibrated with 100 mM potassium phosphate, pH 8.0. The column was washed at a flow rate of 50 ml/hr first with 10 mM potassium phosphate, pH 8.0 and then with 30% ethylene glycol in 10 mM potassium phosphate, pH 8.0. Elution was performed at a flow rate of 50 ml/hr with 80 % ethylene glycol in 10 mM potassium phosphate, pH 8.0. 2 ml fractions were collected and the fractions containing protein (detected by absorbance at 280 nm) were checked for enzyme activity. The active fractions were pooled and dialyzed against 2 mM glycine-NaOH, pH 10.0. The dialyzed protein was diluted 1:1 with 50 mM Bicine-NaOH, pH 8.5 (buffer A) and loaded onto a MonoS HR5/5 (Pharmacia) column, pre-equilibrated with buffer A, using a Superloop (Pharmacia) on a FPLC (Pharmacia) system. The protein-bound-column was washed thoroughly with the buffer A to remove unbound proteins. The protein was eluted using a linear gradient with buffer A to buffer B (50 mM Bicine-NaOH, pH 8.5, 1 M NaCl). The enzyme eluted around 300 mM NaCl as a single peak. The active fractions eluted from the MonoS column were dialyzed overnight against 2 mM glycine, pH 10.0 and concentrated using an Amicon concentrator fitted

with a YM10 membrane (10 kD cutoff). Purity of the protein was checked on a 12% SDS-PAGE gel containing 5 M urea (Lessuisse *et al*, 1993). The protein was > 95 % pure on a Coomassie stained gel (See Fig.4).

Example 2:

Assay of lipase:

Lipase belongs to a class of enzymes known as interfacially active enzymes. These enzymes have very little activity on the substrate monomers but their activity increases dramatically on insoluble substrate such as emulsified triglycerides, monolayers etc. This property makes lipases dissimilar to other enzymes which act on soluble substrate monomers. Triglycerides, natural substrates of lipase are not very convenient to set up simple chromogenic assays. Activity of pure lipases, sometimes, can be monitored by detecting the pH changes using pH-sensitive dyes. However, such assays yield complications when the enzyme source is a lysate and when there are other processes that may alter the pH. P-nitrophenyl esters are most convenient to monitor the activity. Short chain ester, p-nitrophenyl acetate and long chain ester, p-nitro phenyl oleate (PNPO), were synthesized by routine synthetic methods (given below). PNPO is a insoluble ester, was used in our assays using triton X-100 as a solubilizing agent. Triton X-100: PNPO co-micelles showed low back ground hydrolysis and were also stable at elevated temperatures. 96-well plate assays for screening the variants of lipase, though very useful to screen large number of samples, quantitates the activity approximately. All positives obtained in 96-well screens were confirmed in a tube assays, where the number of samples are less and more accurate specific activity calculations could be made.

Synthesis of chromogenic substrates for lipase assays

The following chromogenic substrates were synthesized for lipase assays:

1) p-nitrophenyl oleate
2) p-nitrophenyl stearate
3) p-nitrophenyl caprylate

The fatty acid, N, N'- methyltetrayl biscyclohexamine (dicyclohexylcarbodiimide, DCC), N, N'- dimethylamino pyridine (DMAP), and p-nitrophenol were taken in mole ratios of 1:1:1:2. The fatty acid was taken in a round bottom flask containing 20 ml of dry DCM and a few ml of chloroform. The mixture was stirred for two minutes

followed by addition of DCC. A white precipitate was formed. This was followed by the addition of DMAP. Subsequent addition of p-nitrophenol led to the formation of a yellow precipitate. The reaction vessel was flushed with nitrogen and stirred for 5 hours. The progress of the reaction was monitored by thin layer chromatography. After completion of the reaction, the DCM was evaporated to dryness and the ester was purified by column chromatography (silica gel column, elution with petroleum ether-acetone). The purity and identity of the product was confirmed by ^{1}H-NMR spectroscopy.

Example 3

Lipase assay in 96-well microtitre plates): The colonies obtained from the cloning of the PCR product generated by error-prone PCR were patched on another similar plate and simultaneously inoculated in separate wells of a microtitre plate containing 200 µl 2XYT containing 25 µg/ml chloramphenicol and 0.2 % glucose. The cells were grown for 24 hours in the microtitre plate with continuous shaking at 200 rpm. After 24 hrs, 5 µl culture from each well was taken and added to the corresponding well another microtitre plate containing 200 µl 2XYT supplemented with 25 µg/ml chloramphenicol. After 3 hours of growth the cultures were induced with 1 mM IPTG. After 3 more hours 25 µl of culture was taken from every well into the corresponding wells of two fresh microtitre plates containing 25 µl phosphate buffer pH 7.0. One of the plates was exposed to high temperature for 20 min, cooled on ice for 15 min and then allowed to come to room temperature. The other plate was kept at room temperature. 25 µl of the PNPO-Triton X-100 substrate solution prepared as described above was added to each well. The plates were incubated at 37 °C and absorbance at 405 nm was recorded in an ELISA reader at definite time intervals. The clones showing less than 20 % of the activity of the wild-type protein (or the parent from which it is generated) were removed from further consideration. The residual activity for each clone after exposure to high temperature was calculated. The clones showing highest residual activity were chosen for the next level of screening.

Lipase assays in tubes:: The colonies that showed highest residual activity in the microtire plate level screen were grown for 12 hours in 5 ml 2XYT medium 25 µg/ml chloramphenicol and 0.2 % glucose. 10 ml of 2XYT containing 25 µg/ml chloramphenicol and 0.2 % glucose was inoculated with 100 µl of the overnight grown

culture. After 2.5 hours growth, the cultures were induced with 1.5 mM IPTG and were harvested after another 2.5 hours. The cell pellet was washed with STE and resuspended in 1 ml 0.05 M potassium phosphate buffer pH 7.2. The cell suspension was sonicated with a Branson sonicator with four pulses of 30 sec and 1 min cooling time in between the pulses. The tubes were kept on ice during sonication and cooling of the samples. The sonicated samples were centrifuged at 15,000 rpm for 45 min and the supernatant was used for the assays. The supernatant was divided into four 250 µl aliquots. Three of the aliquots were exposed to higher temperatures and the fourth was kept on ice. The tubes were exposed to high temperatures for 20 min, chilled on ice, centrifuged at 4 °C at 15,000 rpm and then allowed to come to room temperature before assaying for enzymatic activity. The lipase activity in the cell lysates was determined at room temperature in sodium phosphate buffer pH 7.2 by using p-nitrophenyl oleate as substrate. The enzymatic activity was measured by following the change of absorbance at 405 nm with time. Lysates of cells that do not contain the lipase gene but otherwise processed in the same way as mentioned above, were used to determine the background hydrolysis of p-nitrophenyl oleate in *E.coli* cell lysate. The background hydrolysis values were subtracted from the enzymatic activity value. The total protein in the cell lysates was determined by Lowry's method and was used to normalize the activity.

Example 4

Half-lives of thermal inactivation

Exposing the enzymes to higher temperatures and then assaying the activity at room temperature normally assess thermostability of enzymes. At higher temperature the protein denatures and irreversible unfolds. Thermostable enzymes possess additional stabilizing interactions which would make them less susceptible for heat denaturations. The activity remaining is residual activity, which decrease both with increase in temperature or with increase in time at a given temperature. Heat treatment of the purified proteins was carried out in a programmable thermal cycler (GeneAmp PCR system 9700) in 0.2 ml thin-walled PCR tubes to allow precise temperature control of the samples. The proteins were taken at a concentration of 0.05 mg/ml in 0.05 M sodium phosphate buffer, pH 7.0. 25 µl of protein samples were taken in each tube. The proteins were heated for the required time, cooled at 4 °C for 20 min, centrifuged and equilibrated at room temperature before assaying for enzymatic

activity. 20 µl of the heat-treated protein sample was added to 1 ml 0.05 M sodium phosphate, pH 7.2 containing 2 mM p-nitrophenyl acetate. Enzymatic activity was measured at 25 °C by monitoring the rate of increase in absorbance at 405 nm. Typically, inactivation was followed until > 80 % of the activity was lost. Plots of log(residual activity) versus time were linear. Inactivation rate constants (k_{inact}) were obtained from the slope and half-lives were calculated as $t_{1/2} = \log 2/k_{inact}$. The half lives of various mutants obtained were presented in figure (Fig.6 and 7) where the residual activities were measured using PNPA as substrate. The enzyme mutants were exposed to 55 C. In fig.8 data obtained with residual activities with three mutants using olive oil as a substrate was presented. The activities were measured using pH stat equipment. This data demonstrates that the enhancement seen with mutants was independent of the substrate and nature of the assay.

Preparation of substrate stocks:

Appropriate amounts of the insoluble p-nitrophenyl ester and Triton X-100 were weighed out in a glass vial and mixed with a magnetic stirrer till the ester completely dissolved in Triton X-100. Buffer was added slowly while stirring to prepare a 2X stock solution containing 0.4 mM p-nitrophenyl ester and 40 mM Triton X-100. Substrate solutions prepared in this way were optically clear. 100X substrate stocks of the water-soluble p-nitrophenyl acetate were made in acetone and 2 mM p-nitrophenyl acetate was used for each reaction. The reactions were carried out in absence of Triton X-100 and all the measurements to determine kinetic parameters were done with this reaction system.

Example 5

Assay with olive oil (Diagrammatic sketch of breakdown of fats/detergents by lipases, Fig.5)

Assay with the olive oil is performed pH stat equipment. All lipases subsequent to thir activity reduce th pH off the reaction medium by releasing a proton. The decrease in pH could be neutralized by addition of known amounts of alkali. The rate of additon of alkali would represent the activity of the lipase. We have prepared the lipase substrate by mixing gum Arabic (0.5%), olive oil and CaCl2. The mixture was sonicated in a bath till we obtain a uniform emulsion. We have used 10 ml of the substrate for each assay.At the beginning of the asay the pH of the substrate was brought to 8.4 by

addition of alkali. The reaction was started with the addition of 10 microlitres of 1mg/ml enzyme solution. The rate of reaction was calculated from the slopes of amount of alkali vs. time curves.).1 N NaOH was sued as alkali.

Example 6

Methods of generation of variations in the Lipase genes:

The sequence of LipA, whose product is lipase gene of interest in this invention, from *Bacillus subtilis* was published. In Bacillus LipA gene product is secreted into the culture medium owing to the presence of a signal sequence at the N-terminal of the sequence, which aids in its transport out of the cell. Molecular biology of Bacillus species has been well studied and it is a Gram-positive strain. For routine molecular biology techniques such as transformation, cloning, expression etc. Bacillus sp. is less suited compared to E.coli (Sambrook *et al.*, 1989; Hoch *et al.*, 1993). The main difficulty is in transforming the Bacillus sp with the plasmids. The efficiency is lower by several orders of magnitude compared to E.coli. Further, the observed efficiencies are only detectable with electroporation, which is a harsher method. In E. coli the transformation efficiency is higher and reproducible and the choice of plasmids is wide. To perform various gene manipulations, E.coli was used.

The clone pLipA containing the complete lipA gene in pBR322 plasmid was a kind gift from Dr Frens Pierce (Fig.1). The lipase gene along with the region coding for the signal sequence was amplified with primers For1 (forward primer) (5'-GGAGGATCATATGAAATTTGTAAAAA-3') and Rev1 (reverse primer) (5'-CCCGGGATCCATTGTCCGTTACC-3'). The primers contained engineered Nde1 and Bam H1 sites respectively. The ATG of the Nde1 site in For1 coincided with the natural start codon of the lipase and the BamH1 site was beyond the natural stop codon. The amplified product was digested with Nde 1 and Bam H1 and cloned into the Nde 1-BamH1 sites of the plasmid pET-21b yielding the plasmid pET-lipwt (Fig.2) . The lipase gene coding for the mature protein was amplified from pET-lipwt by using primers PREcoR I (forward primer) (5'-CGTCAGCGAATTCCGCTGAACACAT-3') and PRBamH I (reverse primer) (5'-GCGGGAAGGATCCGAATTCGAGCT-3'). The primers had an engineered EcoR I and BamH I site respectively. The amplified product was cleaved by EcoR I and BamH I and cloned into the EcoR1-BamH1 sites of the plasmid pJO290. This construct (pJ0290lip) was used for screening thermostable

mutants (Fig.3). The *E.coli* strain JM109 was used for all the screening steps and all media contained 0.2 % glucose unless otherwise mentioned. This system was chosen because it allows low-level, controlled and inducible expression of the gene product in *E.coli*, which is necessary to prevent the reported toxicity of the protein to *E.coli* and to prevent complications from *in vivo* insolubility of this highly hydrophobic and aggregation-prone protein.

Methods of random mutagenesis:

The critical step in the invention is in the ability to create variations in the gene. The variation generated should be "sufficient' to yield functional variants. Enzymes have evolved over millions of years of evolution and in the process the enzymes may have tested and avoided deleterious mutations and also tested and incorporated beneficial mutations. It is also believed that most of the gene mutations would be silent i.e., they do not bring about a change in amino acid sequence. In random mutagenesis protocols, it is essential to obtain variations in the gene sequence that result in non-silent mutations and excess of variations, wherein the gene product would be non-functional or may not form. Error-prone PCR based mutagenesis protocols need to be optimized to obtain sufficient variation in the activity of the lipase. The success of the directed evolution protocols strongly depends on the control of this variable. The protocols used in the present example were modifications of the published procedures. The lipase gene was mutagenised by error-prone PCR (Cadwell and Joyce, 1992). Primers JOF (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and JOR (5'-TGACACAGGAAACAGCTATGAC-3') flank the gene beyond the Eco R1 and Bam H1 sites present on the plasmid. Error-prone PCR was carried out in a 100 µl reaction volume containing 20 femtomoles of the plasmid pJO290-lip, 50 pmoles each of primers JOF and JOR, 100 mM Tris.Cl (pH 8.3 at 25 °C), 500 mM KCl, 0.1 % gelatin (w/v), 7 mM $MgCl_2$, 0.25 mM $MnCl_2$, 1 mM each of dTTP and dCTP, 0.2 mM each of dATP and dCTP and 5 units Taq DNA polymerase. After an initial denaturation of 3 min at 94 °C, the following steps were repeated for 30 cycles in a thermal cycler: 1 min at 94 °C, 1 min at 45 °C and 1 min at 72 °C. The amplified product was precipitated with ethanol, eluted from a 1 % agarose gel and digested with EcoR I and BamH I. The digested product was again eluted from a 1 % agarose gel and ligated with pJO290 digested with EcoR I and BamH I. The ligation mix was transformed into *E.coli* JM109

and selection was done on LB-agar supplemented with 25 μg/ml chloramphenicol and 0.2 % glucose.

Site-directed mutagenesis

Site directed mutagenesis was carried out on the lipase gene cloned in pET-21b by a modified PCR technique (Chen and Arnold, 1991). For each substitution an oligonucleotide containing the desired mutation was used as the primer (mismatch primer) to initiate chain extension between the 5' and 3' PCR primers. In the first PCR, the mismatch primer and the 3' primer were used to generate a DNA fragment containing the new base substitution. The fragment was separated from the template and primers by agarose gel electrophoresis, purified and used as the new 3' primer in a second PCR with the 5' primer to generate full length product, which was cloned into pET-21b for expression of the mutant protein.

Example 7

Recombination of the clones obtained in generation 2

The mutant Gene sequence 5 was created from the clone 2-8G10 and wt by using the unique restriction site Hae II at position 910 of the lipase gene. The genes coding for the two proteins were amplified by PCR using the T7 promoter and terminator primers. The PCR products were purified by gel extraction and digested with Hae II and Nde I. The upper and lower bands correspond to the C-terminal and N-terminal regions of the protein, respectively. The upper band from clone 2-8G10 and the lower one from the wild-type protein were eluted. The higher molecular weight fragment was digested with BamH I and purified. A three point ligation containing the Nde I-Hae II fragment (from the wt), the Hae II-BamH I fragment (from 2-8G10) and pET-21b cut with Nde I and Hae II was set up, the ligation mix transformed into DH5α and the positives selected. The sequence of the gene was confirmed by DNA sequencing.

The mutant Gene sequence 6 (triple mutant) was created by site-directed mutagenesis on the Gene sequence 5 template using the mutagenic primer PROLF: 5'- GGC AAG GCG CCT CCG GGA ACA GAT- 3' to incorporate a codon change CTT → CCT that led to L114P change in the amino acid sequence. The sequences of all the genes were confirmed by automated DNA sequencing.

Example 8

Enzyme kinetics

All kinetic measurements were made using a thermostatted spectrophotometer using the water-soluble substrate p-nitrophenyl acetate. Initial rates of hydrolysis of p-nitrophenyl acetate at various concentrations were determined at 25 °C in sodium phosphate buffer pH 7.2. The values for K_M and k_{cat} were derived from the corresponding Lineweaver-Burke plots. The kinetic parameters obtained with wild type and the mutants was presented in fig. 9.

Example 9

Activity of lipase and its mutants in the presence of organic solvents

The activity of the lipasea nd its mutants was checked in the presence of various solvents. The organic solvents tested were acetonitrile, isopropanol, dimethyl sulfoxide and dimethyl formamide. The activity assay was performed using PNPA as a substrate. The substrate (2 mM) was dissolved in various percents (v/v) of the organic solvent in buffer (50 mM pH 8.0) and the reaction was started with the addition of lipase at a concentration of 0.246 mg/ml. The activity was monitored as n increase in absorption at 410 nm and the specific activity was calculated using the initial slopes of the curve. In fig 10 and 11 the data obtained with acetonitrile is presented.

The preceding examples demonstrate the usefulness of the present invention in generating, identifying and isolating lipases which have improved stability and/or ester hydrolysis activity at higher temperature in organic media relative to the natural enzyme.

Example 10

Wild type lipase or control lipase activity in presence of various of detergents. (Fig.12)

Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the disclosures herein are exemplary only and that various other alternations, adaptations and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein.

SEQUENCE LISTING

All gene sequences are of 552 base pairs of linear single stranded DNA.

All protein sequences are of 181 amino acids in length and have enzymatic properties. It should be noted that the sequences presented here are identical to the published lip A sequence except for the first amino acid , methinine present in our sequence. The sequence purified and crysallized in literature does not have the N-terminal methionine.

GENERAL INFORMATION

APPLICANT: COUNCIL OF SCIENTIFIC AND INDUSTRAIL RESEARCH
TITLE: STABLE GENE VARIANTS OF LIPASES
NUMBER OF SEQUENCES 14
CORRESPONDING ADDRESS: Centre for Cellular and Molecular Biology, Uppal Road, Hyderbad-500 007,INDIA

Protein Seq ID No. 1
INFORMATION FOR SEQ ID NO:1
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:1

MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVNSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:1
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccaaat caaaagattt tatacacatc catttacagc agtgccgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtcaacag cctgattaaagaagggctga acggcgggggg ccagaatacg aattaatga
ORGANISM: *Bacillus subtilis*

IMMEDIATE: NATURAL

NAME/KEY: Natural

SEQUENCE ID No.1

Protein Seq ID No. 2
INFORMATION FOR SEQ ID NO:1
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:2

MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:2
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccaaat caaaagattt tatacacatc catttacagc agtgccgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.2

Protein Seq ID No.3
INFORMATION FOR SEQ ID NO:3
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:3
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGVKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPNQKILYTSIYSSDDMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:3
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
(iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgtgaaaaaagtggatattg tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccaaat caaaagattt tatacacatc catttacagc agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.3

Protein Seq ID No.4
INFORMATION FOR SEQ ID NO:4
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGTKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKAPPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:4
- (i) SEQUENCE CHARACTERISTICS:

#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tacgaaaaaagtggatattg tcgctcacag catgggggge gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgcct ccgggaac agatccaaat caaaagattt tatacacatc catttacagc agtgccgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga

ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.4

Protein Seq ID No.5

INFORMATION FOR SEQ ID NO:5
- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:5

MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPNQKILYTSIYSSDDMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:5
- (i) SEQUENCE CHARACTERISTICS:

#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg tcgctcacag catgggggge gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccaaat caaaagattt tatacacatc catttacagc agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga

ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.5

Protein Seq ID No.6
INFORMATION FOR SEQ ID NO:6
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:6
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKAPPGTDPNQKILYTSIYSSDDMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:6
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga
gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac
aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg
tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc
aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgccct ccgggaac agatccaaat
caaaagattt tatacacatc catttacagc agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag
aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag
cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.6

Protein Seq ID No.7
INFORMATION FOR SEQ ID NO:7
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:7
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGASNVQIH
GGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:7
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga
gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac
aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg
tcgctcacag catgggggge gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc
aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccaaat
caaaagattt tatacacatc catttacagc agtgccgata tgattgtcat gaattactta tcaagattag
atggtgctagtaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag
cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.7

Protein Seq ID No.8
INFORMATION FOR SEQ ID NO:8
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:8
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKAPPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:8
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga
gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac
aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg
tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc
aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgcccc ccgggaac agatccaaat
caaaagattt tatacacatc catttacagc agtgccgata tgattgtcat gaattactta tcaagattag atggtgctag
aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag
cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.8

Protein Seq ID No.9
INFORMATION FOR SEQ ID NO:9
- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: enzyme

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:9
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKADIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:9
- (i) SEQUENCE CHARACTERISTICS:

#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga
gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac
aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagcggatattg
tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc
aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccaaat
caaaagattt tatacacatc catttacagc agtgccgata tgattgtcat gaattactta tcaagattag atggtgctag
aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag
cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.9

Protein Seq ID No.10
INFORMATION FOR SEQ ID NO:10
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:10
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLVGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:10
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga
gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac
aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg
tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc
aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccaaat
caaaagattt tatacacatc catttacagc agtgccgata tgattgtcat gaattactta tcaagatta gttggtgctag
aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag
cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.10

Protein Seq ID No.11
INFORMATION FOR SEQ ID NO:11
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:11

MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGVKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKALPGTDPDQKILYTSIYSSDDMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:11
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga
gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac
aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgtgaaaaaagtggatattg
tcgctcacag catgggggggc gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc
aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgc ttccgggaac agatccagat
caaaagattt tatacacatc catttacagc agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag
aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag
cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.11

Protein Seq ID No.12
INFORMATION FOR SEQ ID NO:12
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: enzyme
(iii) SEQUENCE DESCRIPTION: SEQ ID NO:12
MAEHNPVVMVHGIGGASFNFAGIKSYLVSQGWSRDKLYAVDFWDKTGTNY
NNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYYIKNLDGGNKVANV
VTLGGANRLTTGKAPPGTDPDQKILYTSIYSSDDMIVMNYLSRLDGARNVQI
HGGHIGLLYSSQVYSLIKEGLNGGGQNTN

CORRESPONDING GENE SEQUENCE OF PROTEIN SEQ ID NO:12
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 552
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO

atggctgaacaca atccagtcgt tatggttcacggtattggag gggcatcatt caattttgcg ggaattaaga
gctatctcgt atctcagggctggtcgcggg acaagctgta tgcagttgat ttttgggaca agacaggcac
aaattataacaatggaccgg tattatcacg atttgtgcaa aaggttttag atgaaacggg tgcgaaaaaagtggatattg
tcgctcacag catgggggge gcgaacacac tttactacat aaaaaatctggacggcggaa ataaagttgc
aaacgtcgtg acggttggcg gcgcgaaccg tttgacgacaggcaaggcgcect ccgggaac agatccagat
caaaagattt tatacacatc catttacagc agtgacgata tgattgtcat gaattactta tcaagattag atggtgctag
aaacgttcaaatccatggcg ttggacacat cggccttctg tacagcagcc aagtctacag
cctgattaaagaagggctga acggcggggg ccagaatacg aattaatga
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Aritifical

SEQUENCE ID No.12

INFORMATION FOR SEQ ID NO:13
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
(iii) HYPOTHETICAL: NO

5'-CGCCAGGGTTTTCCCAGTCACGAC-3'
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Oligonucleotide

SEQUENCE ID No.13

INFORMATION FOR SEQ ID NO:14
- (i) SEQUENCE CHARACTERISTICS:
#pairs (A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
(iii) HYPOTHETICAL: NO

5'- TGACACAGGAAACAGCTATGAC-3'
ORGANISM: Artifical

IMMEDIATE: Aritifical

NAME/KEY: Oligonucleotide

SEQUENCE ID No.14

REFERENCES:

1. Karsherkoff, A. and Ladenstein, R. *TIBS* (2001) 26, 550-556
2 Kumar,S, Tsai,C-J and Nussinov,R. (2000) *Protein Engg.* 13: 179-191.
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We Claim:

1. Novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants having SEQ ID No. 2 of molecular wt 19443, SEQ ID No. 3 of molecular wt 19515 SEQ ID No. 4 of molecular wt 19456.9, SEQ ID No.5 of molecular wt.19487and SEQ ID No.6 of molecular wt. 19470.9
2. Novel gene variants as claimed in claim 1, wherein said gene variants are thermostable in the temperature range of about 45 to 95°C.
3. Novel gene variants as claimed in claim 2, wherein said gene variants are highly thermostable at the temperature in the range of about 55 to 90°C.
4. Novel gene variants as claimed in claim 1, wherein $T_{1/2}$ value is in the range of 6 to 685.
5. Novel gene variants as claimed in claim 1, wherein $T_{1/2}$ value is in the range of 7 to 677.
6. Novel gene variants as claimed in claim 1, wherein Km value is in the range of 0.50 to 2.5 mM.
7. Novel gene variants as claimed in claim 1, wherein Km value is in the range of 0.63 to 1.96 mM.
8. Novel gene variants as claimed in claim 1, wherein k_{cat} value is in the range of 4.5×10^{-2} to 8.5×10^{-2} min^{-1}.
9. Novel gene variants as claimed in claim 1, wherein k_{cat} value is in the range of 5×10^{-2} to 8.1×10^{-2} min^{-1}.
10. Novel gene variants as claimed in claim 1, wherein k_{cat}/K_m value is in the range of 4×10^{-2} to 10×10^{-2} min^{-1}.
11. Novel gene variants as claimed in claim 1, wherein k_{cat}/K_m value is in the range of 4.1×10^{-2} to 9.7×10^{-2} min^{-1}.
12. Novel gene variants as claimed in claim 1,wherein said gene variants are resistant to organic solvents selected from group of acetonitrile, isopropanol, dimethyl sulfoxide and dimethyl formide.
13. Novel gene variants as claimed in claim 4, wherein organic solvent used is acetonitrile.
14. Novel gene variants as claimed in claim 1, wherein residual activity of the gene variants is in the range of 25 to 100 % in presence of acetonitrile.

15. Novel gene variants as claimed in claim 1, wherein residual activity of the gene variants is in the range of 28.7 to 85.5% in presence of acetonitrile
16. Novel gene variants as claimed in claim 1, wherein the gene variants have inherent ability to withstand high pH in the range of 9 to 13; ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof.
17. Novel gene variants as claimed in claim 16, wherein the gene variants have inherent ability to withstand high pH in the range of 9 to 13; ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof.
18. An expression system for novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants said expression system comprising of having SEQ ID No. 2 of molecular wt 19443, SEQ ID No. 3 of molecular wt 19515, SEQ ID No. 4 of molecular wt 19456.9, SEQ ID No.5 of molecular wt. 19487 and SEQ ID No.6 of molecular wt 19470.9 present in the vector pJO290.
19. An expression system as claimed in claim in 18, wherein said gene variants are thermostable in the temperature range of about 45 to 95°C.
20. An expression system as claimed in claim in 19, wherein said gene variants are highly thermostable at the temperature of about 55 to 90°C.
21. An expression system as claimed in claim in 18, wherein $T_{1/2}$ value is in the range of 6 to 685.
22. An expression system as claimed in claim in 21, wherein $T_{1/2}$ value is in the range of 7 to 677.
23. An expression system as claimed in claim in 18, wherein Km value is in the range of 0.50 to 2.5 mM.
24. An expression system as claimed in claim in 23, wherein Km value is in the range of 0.63 to 1.96 mM.
25. An expression system as claimed in claim in 18, wherein k_{cat} value is in the range of 4.5×10^{-2} to 8.5×10^{-2} min^{-1}.
26. An expression system as claimed in claim in 25, wherein k_{cat} value is in the range of 5×10^{-2} to 8.1×10^{-2} min^{-1}.

27. An expression system as claimed in claim in 18, wherein k_{cat}/K_m value is in the range of 4×10^{-2} to 10×10^{-2} min^{-1}.

28. An expression system as claimed in claim in 27, wherein k_{cat}/K_m value is in the range of 4.1×10^{-2} to 9.7×10^{-2} min^{-1}.

29. An expression system as claimed in claim in 18, wherein said gene variants are resistant to organic solvents selected from group of acetonitrile, isopropanol, dimethyl sulfoxide and dimethyl formide.

30. An expression system as claimed in claim in 29, wherein organic solvent used in acetonitrile.

31. Novel gene variants as claimed in claim 18, wherein residual activity of the gene variants is in the range of 25 to 100 % in presence of acetonitrile.

32. Novel gene variants as claimed in claim 31, wherein residual activity of the gene variants is in the range of 28.7 to 85.5% in presence of acetonitrile

33. Novel gene variants as claimed in claim 18, wherein the gene variants have inherent ability to withstand high pH in the range of 9 to 13; ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof.

34. Novel gene variants as claimed in claim 33, wherein the gene variants have inherent ability to withstand high pH in the range of 9 to 13; ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof.

35. A method of preparing an expression system of novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants having SEQ ID No. 2 of molecular wt 19443, SEQ ID No. 3 of molecular wt 19515, SEQ ID No. 4 of molecular wt 19456.9, SEQ ID No.5 of molecular wt. 19487 and SEQ ID No.6 of molecular wt 19470.9 said method comprising the steps of :

    (a) isolating and purifying lipase gene from *Bacillus subtilis*, ,

    (b) cloning lipase gene isolated in step (a) in vector pJO290,

    (c) generating gene variants from lipase gene isolated in step (a) by random mutagensis and site-directed mutagenesis using forward primer JOF having SEQ ID No.13 and reverse primer JOR having SEQ ID No. 14,

(d) cloning the gene variants obtained in step (c) in plamsid vector pJO290, and

(e) ligating the cloned gene variants of step (d) in *E.coli* JM109.

36. A method as claimed in claim 35, wherein said gene variants are thermostable in the temperature range of about 45 to 95°C.

37. A method as claimed in claim 36, wherein said gene variants are highly thermostable in the temperature range of about 55 to 90°C.

38. A method as claimed in claim 35, wherein $T_{1/2}$ value is in the range of 6 to 685.

39. A method as claimed in claim 38, wherein $T_{1/2}$ value is in the range of 7 to 677.

40. A method as claimed in claim 35, wherein Km value is in the range of 0.50 to 2.5 mM.

41. A method as claimed in claim 40, wherein Km value is in the range of 0.63 to 1.96 mM.

42. A method as claimed in claim 35, wherein k_{cat} value is in the range of 4.5×10^{-2} to 8.5×10^{-2} min^{-1}.

43. A method as claimed in claim 42, wherein k_{cat} value is in the range of 5×10^{-2} to 8.1×10^{-2} min^{-1}.

44. A method as claimed in claim 35, wherein k_{cat}/K_m value is in the range of 4×10^{-2} to 10×10^{-2} min^{-1}.

45. A method as claimed in claim 44, wherein k_{cat}/K_m value is in the range of 4.1×10^{-2} to 9.7×10^{-2} min^{-1}.

46. A method as claimed in claim 35, wherein said gene variants are resistant to organic solvents selected from group of acetonitrile, isopropanol, dimethyl sulfoxide and dimethyl formide.

47. A method as claimed in claim 46, wherein organic solvent used in acetonitrile.

48. A method as claimed in claim 35, wherein residual activity of the gene variants is in the range of 25 to 100 % in presence of acetonitrile.

49. A method as claimed in claim 48, wherein residual activity of the gene variants is in the range of 28.7 to 85.5% in presence of acetonitrile

50. A method as claimed in claim 35, wherein the gene variants have inherent ability to withstand high pH in the range of 9 to 13;

51. A method as claimed in claim 34, wherein the gene variants have inherent ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof.

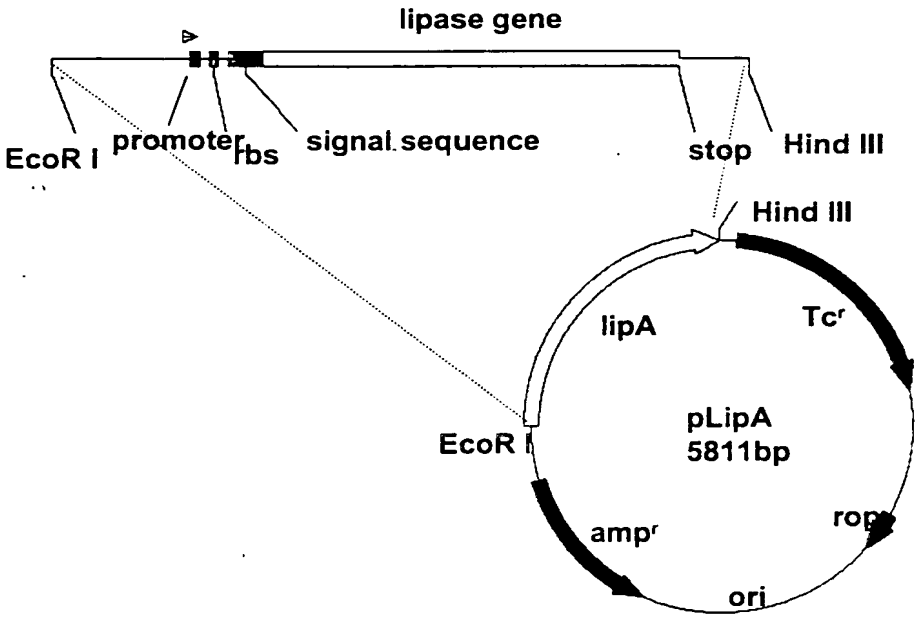
52. A method as claimed in claim 50, wherein the gene variants have inherent ability to withstand high pH in the range of 9 to 13; ability to withstand damaging surfactants and enzymes comprising groups of linear alkyl benzene sulfonates, proteases and compounds thereof.

53. Novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants substantially as herein described with reference to examples and drawings accompanying this specification.

Dated this 30th day of January 2004

Dr. R.V.P. SINHA Scientist, IPMD CSIR

Fig.1


lipase gene
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promoter
rbs
signal sequence
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Hind III
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lipA
Tc^r
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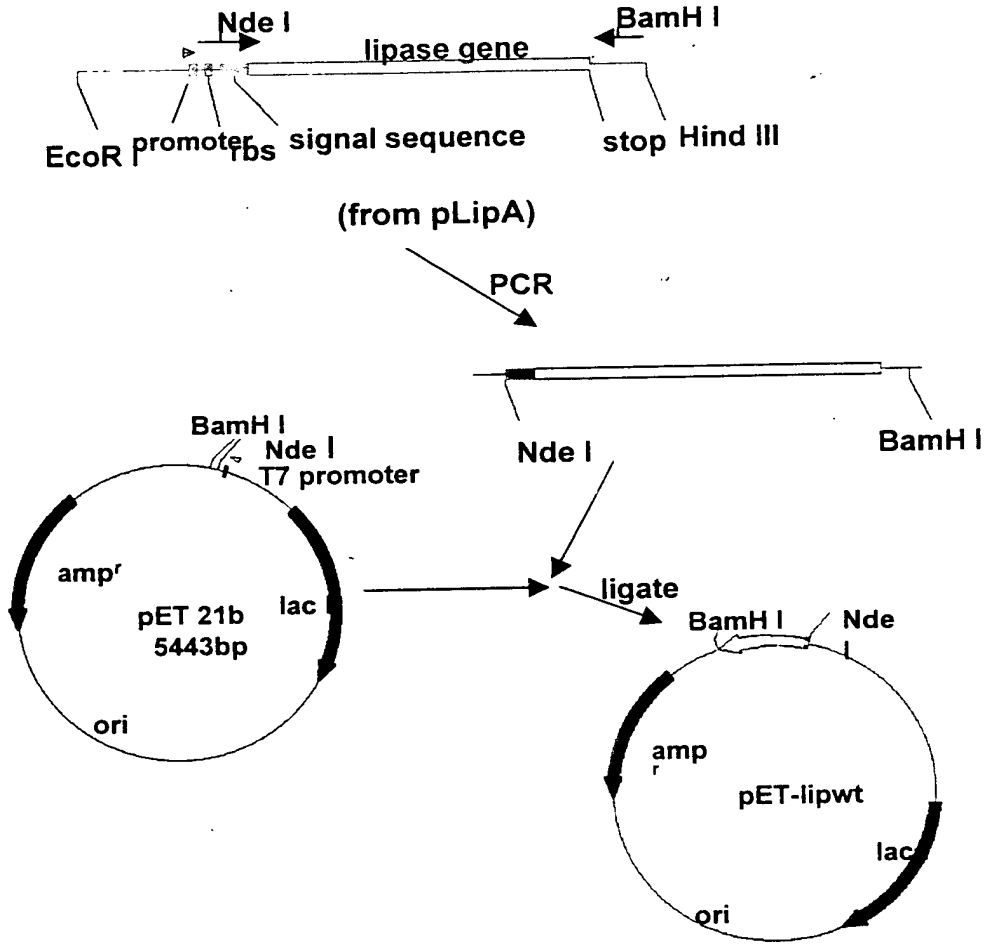
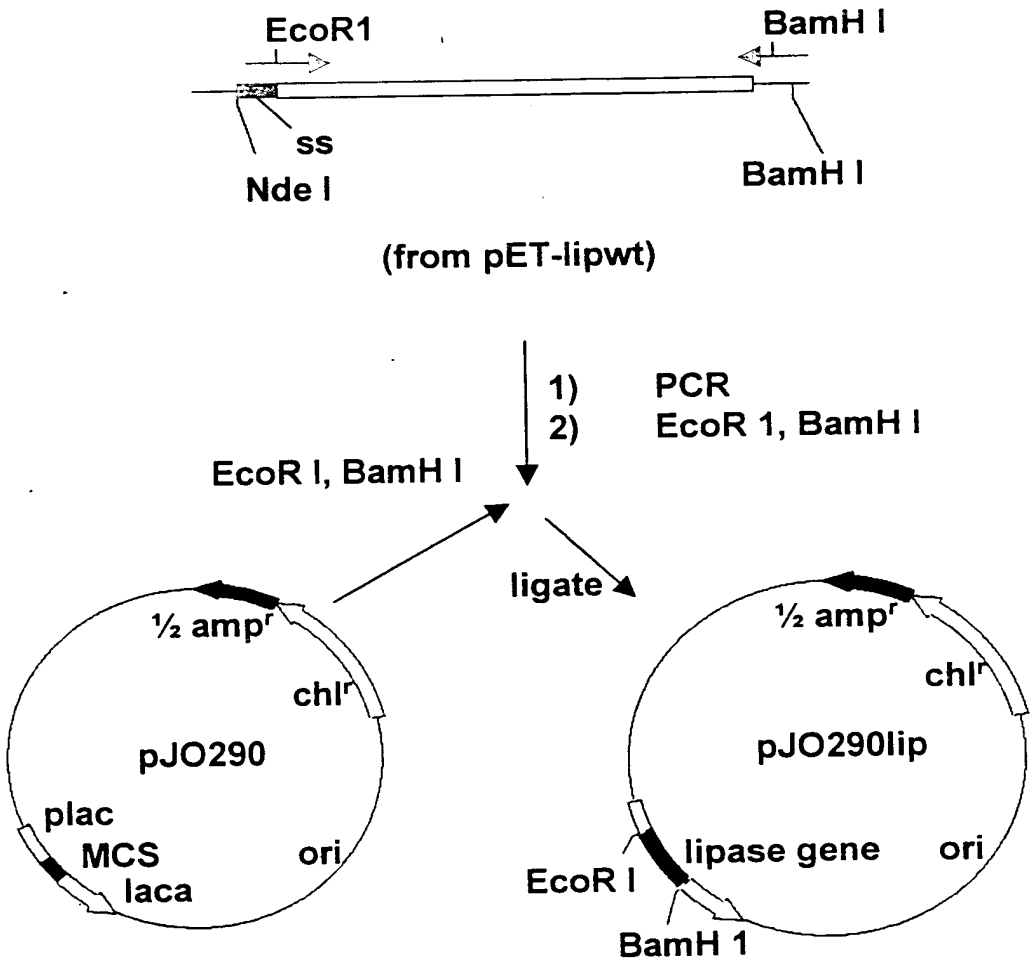
amp^r
ori

Fig.2



(RVP Sinha)

Fig.3


EcoR1
BamH I
ss
Nde I
BamH I
(from pET-lipwt)
1) PCR
2) EcoR 1, BamH I
EcoR I, BamH I
ligate
½ ampr
chlr
pJO290
plac
MCS
laca
ori
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chlr
pJO290lip
lipase gene
ori
EcoR I
BamH 1

Fig.4

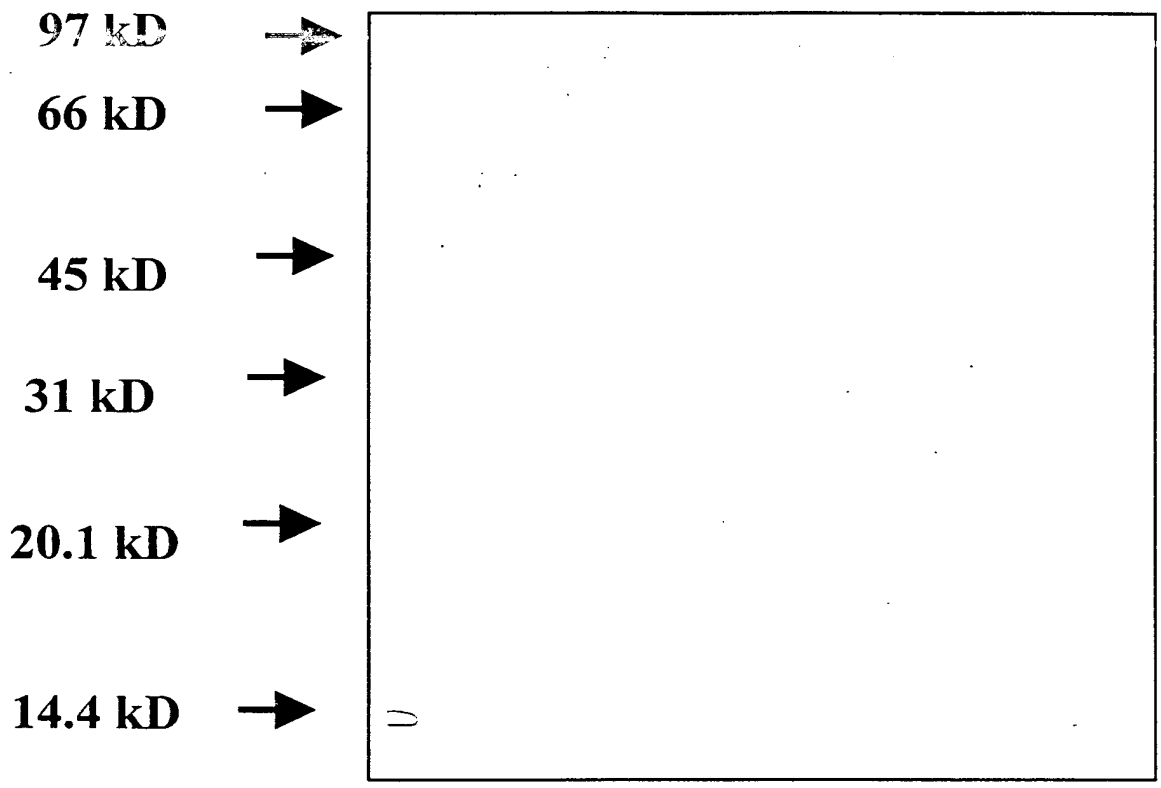


Fig.5

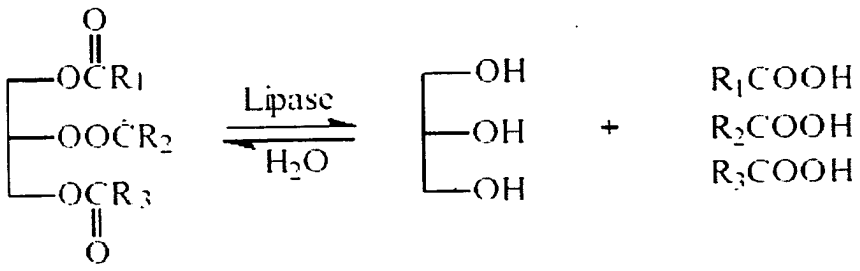


Fig.6.

The residual activity of the proteins wt (○), SEQ ID No. 2 (▲),SEQ ID No. 3 (◆),SEQ ID No. 4(□),SEQ ID No. 5 (●),SEQ ID No. 6(■) plotted against the time of exposure at 55 ° C, pH 7.

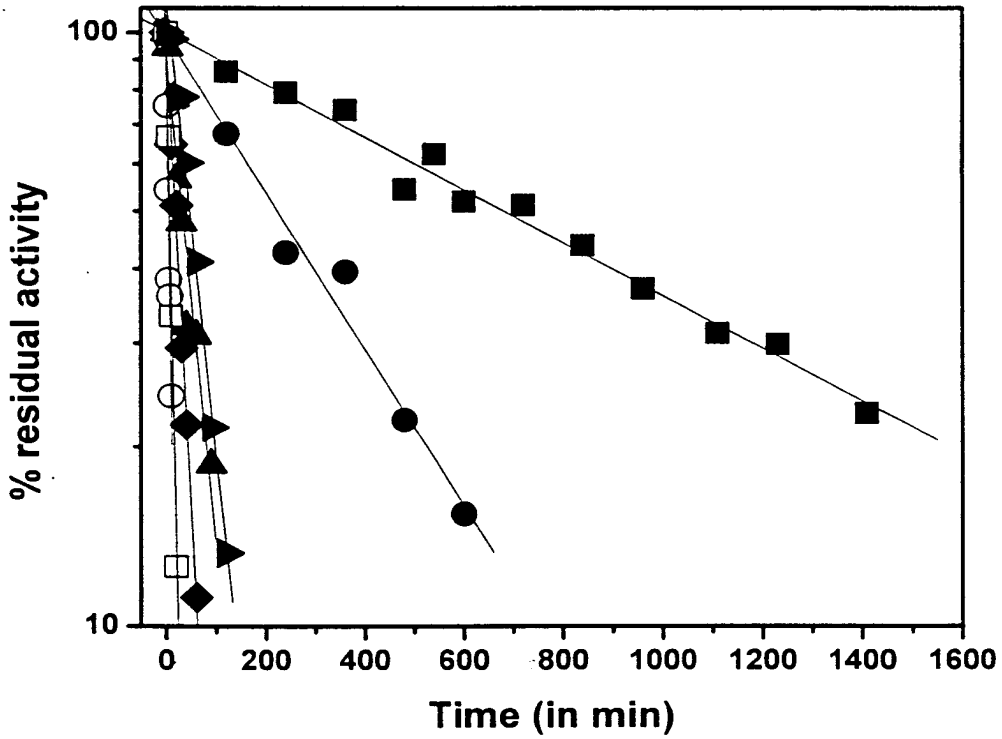


Fig.7

SEQ ID No. 6		SEQ ID No. 5		SEQ ID No. 1		SEQ ID No. 3		SEQ ID No. 4		SEQ ID No. 2	
Time,min	Activity	Time,min	Activity	Time,min	Activity	Time,min	Activity	Time,min	Activity	Time,min	Activity
0	100	0	100	0	100	0	100	0	100	0	100
120	85.88	120	67.52	0.5	97.65	10	64.75	5	66.7466	2.5	94.5
240	79.2133	240	42.635	1.25	75.3	20	51.1233	10	7	7.833	77
360	3	360	39.58	2.5	54.25	30	3	20	33.3333	15	77.6
480	74.115	480	22.205	5	38.4	40	29.45	30	3	20	56.54
540	54.415	540	--	7.5	36.02	60	21.81		12.5933	30	47.9
600	62.354	600	15.425	10	24.4		11.1766		3	40	32.03
720	51.94						7		5.54333	60	30.9
840	51.18									90	18.66
960	43.82										
1110	37.055										
1230	31.1735										
1410	29.85										
	22.79										

Fig.8

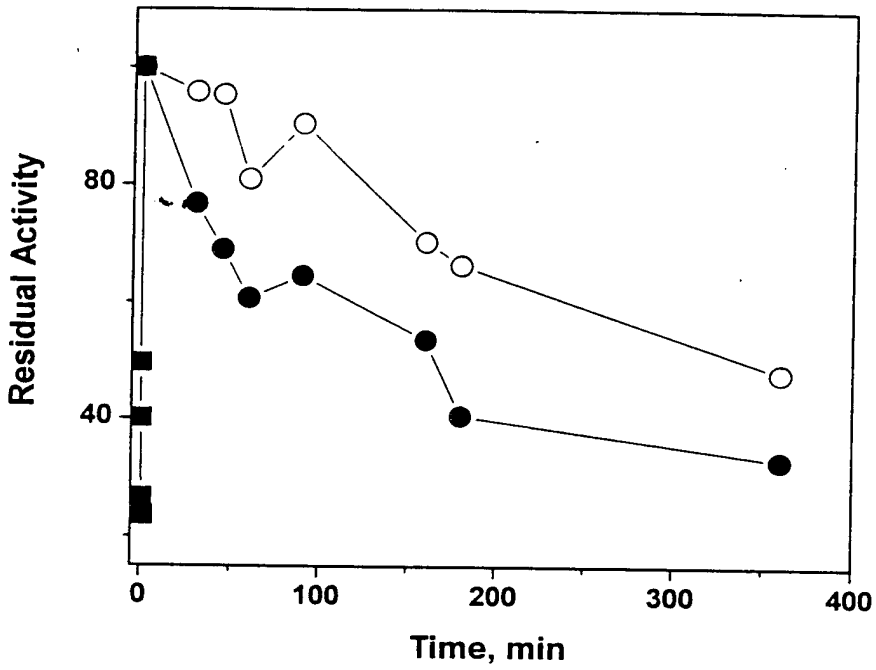


Fig.9.

Kinetic parameters for the wild-type lipase and the thermostable mutants

Clone	K_m, mM	k_{cat} X 10^{-2}, min^{-1}	k_{cat}/K_m X 10^{-2}, $mM^{-1}.min^{-1}$	T ½, min (55°C, pH 7.0)
SEQ ID No.1 (Wild lipases)	0.97	5.2	5.4	2.5
SEQ ID No. 2	1.03	5.0	4.85	25.4
SEQ ID No. 3	0.69	5.5	8.0	18.9
SEQ ID No. 4	0.63	6.1	9.7	7.0
SEQ ID No. 5	1.22	6.8	5.6	228.0
SEQ ID No. 8	1.45	7.5	5.2	46.1
SEQ ID No. 6	1.96	8.1	4.1	677.0

.Fig.10

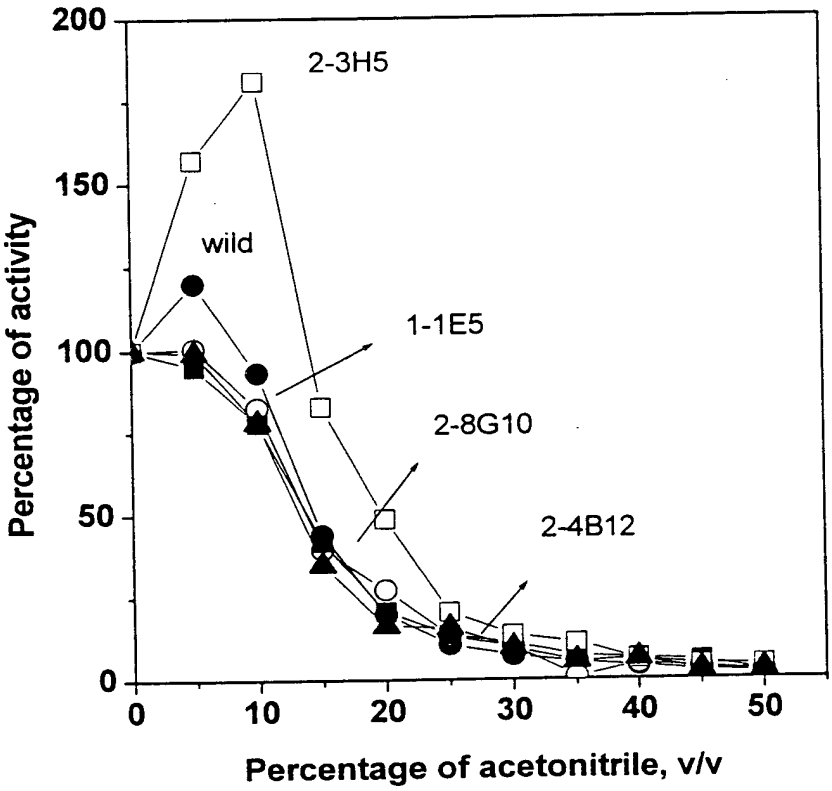
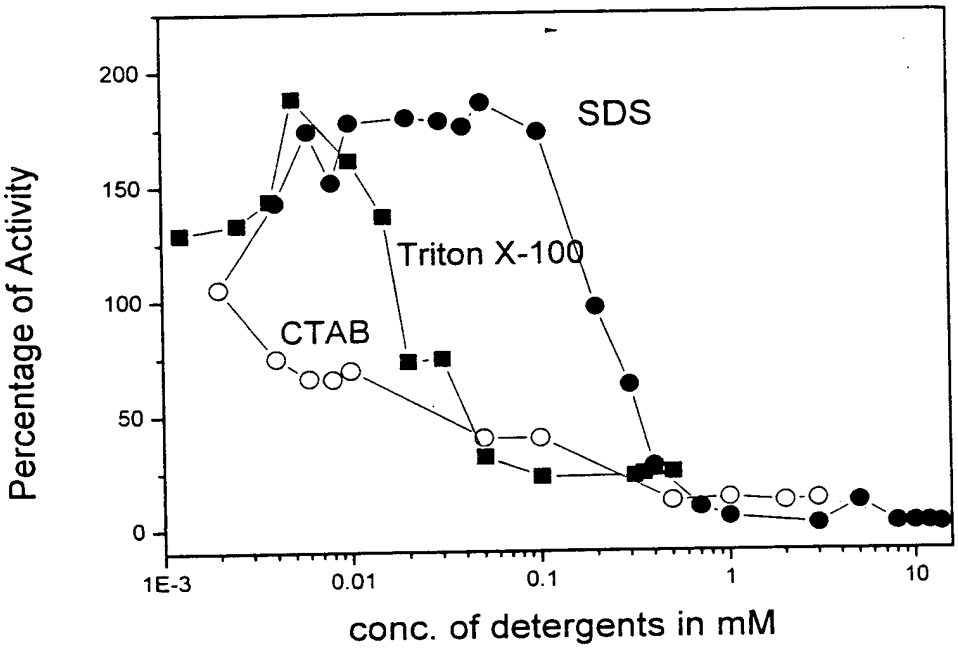

200
150
100
50
0
Percentage of activity
2-3H5
wild
1-1E5
2-8G10
2-4B12
0
10
20
30
40
50
Percentage of acetonitrile, v/v

Fig.11

	Residual activity (as Percent of control) at 20 % Acetonitrile (v/v)
SEQ ID No. 1	22
SEQ ID No. 2	28.7
SEQ ID No. 3	61.6
SEQ ID No. 4	85.5
SEQ ID No. 7	52.3

Fig.12


Percentage of Activity
200
150
100
50
0
1E-3
0.01
0.1
1
10
SDS
Triton X-100
CTAB
conc. of detergents in mM